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SCREENING PROCEDURES FOR EXPERIMENTAL CANCER CHEMOTHERAPY

BY

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SCREENING PROCEDURES FOR EXPERIMENTAL CANCER CHEMOTHERAPY*

Consulting Editor
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* This series of papers is the result of a conference on *Screening Procedures for Experimental Cancer Chemotherapy* held and supported conjointly by The New York Academy of Sciences and the Cancer Chemotherapy National Service Center of the National Cancer Institute, Public Health Service, Bethesda, Md., on March 13, 14, and 15, 1958.

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INTRODUCTION

By K. M. Endicott
Cancer Chemotherapy National Service Center, National Cancer Institute, Public Health Service, Bethesda, Md.

For several reasons, the Cancer Chemotherapy National Service Center has joined with The New York Academy of Sciences in sponsoring this monograph. One of the most important, of course, is that the Service Center, through the activities of its Screening Panel, is supporting a very large screening program in which more than 40,000 materials per year are being tested at a cost of several million dollars. It is therefore important that the methods used be the best available. Along with this large routine screening program, the Service Center also is sponsoring a number of projects that deal with a search for better screening methods. In planning such projects, the Screening Panel must keep abreast of developments in the field. A major purpose of the conference on which this monograph is based was to provide the Service Center with a review of the state of knowledge in the field of anticancer screening.

Another major objective in sponsoring the conference was to provide an opportunity for investigators in this rapidly moving field to meet in order to exchange views and information. The large number of resulting papers presented here will be of value to the many interested investigators throughout the world who were unable to attend the conference.

Part I. Microbiological Studies

INTRODUCTORY REMARKS

By Esmond E. Snell
University of California, Berkeley, Calif.

For several years the National Cancer Institute, Public Health Service, Bethesda, Md., has included on certain of its panels one or more individuals who were entirely outside the field of cancer research, in the hope that their innocence—not to say ignorance—in the field might lead them to suggest novel approaches to certain of its problems. It was with this principal qualification that I accepted temporary appointment to the Screening Panel of the Cancer Chemotherapy National Service Center about two years ago. During the past two decades, as is well known, studies of microbial nutrition and metabolism have contributed enormously to our knowledge of the vitamins and cofactors required for the growth of higher animals, of the metabolic reactions involved in growth processes, and of the metabolite-antimetabolite phenomenon which is the principal basis for hopes that a rational approach to cancer chemotherapy can be made. Many of us felt that if an extensive off-the-shelf screening program was to be initiated, microorganisms would be among the more promising of possible test systems, for the limited successes achieved to date in cancer chemotherapy have, with few exceptions, been with compounds (for example, antimetabolites related to such substances as folic acid and purine bases) first studied because of their effects on bacterial growth.

Despite limited studies, no one has really known how effective microorganisms might be as screening agents for compounds intended for use in mammalian tissues. The Screening Panel of the Cancer Chemotherapy National Service Center therefore undertook an extensive comparison of the toxicities of a selected series of 200 test compounds for a series of different test organisms that included aerobic and anaerobic bacteria, yeasts, and fungi. The results of these tests were compared with those obtained with the same compounds in human tissue cultures and in a variety of animal tumors. These studies with microorganisms are described by George Foley and his co-workers in the first paper of Part I. Foley is one of the principal investigators participating in these studies. Following this paper, we turn to more exploratory aspects of the use of microorganisms in study of compounds of interest in cancer chemotherapy.

A COMPARATIVE STUDY OF THE USE OF MICROORGANISMS IN THE SCREENING OF POTENTIAL ANTITUMOR AGENTS*

By G. E. Foley,† R. E. McCarthy, V. M. Binns

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INTRODUCTION

The selection of compounds of possible interest as antitumor agents presents a major problem to those concerned with the chemotherapy of neoplastic disease in man. The ideal method for the selection of such compounds must not only be simple, rapid, inexpensive, and adaptable to large-scale operation, but should detect the maximum number of antitumor agents with a rate of "false positives" sufficiently low not to invalidate the use of the method as a screening procedure. The members of the Subcommittee on Microbiology, Screening Panel, Cancer Chemotherapy National Service Center (CCNSC), National Cancer Institute, Public Health Service, Bethesda, Md., have been engaged in the comparative study of several microbiological assay systems to determine the extent of the usefulness of such methods for the detection of potential antitumor agents.

The application of bacterial, fungal, and protozoan bioassay methods to this problem is not a new approach; however, the majority of earlier studies have been based upon the use of a single bacterial (or other) bioassay system for this purpose.[1] It should not be expected that any single *in vitro* system will detect all compounds exhibiting antitumor activity in any one or more diverse *in vivo* experimental tumor systems; this requires no elaboration. In the expectation that any *in vitro* microbiological screening procedure must be based upon multiple bioassay systems covering a variety of diverse metabolic pathways, these collaborative studies were organized to include sixteen microbial systems, using bacteria, fungi, and protozoa as the assay microorganisms. Certain combinations of some of these systems have been used extensively for some time,[2] others have had some use,[3] and still others have not been used to any extent for the detection of potential antitumor agents. Insofar as we are aware, the present studies encompass a larger variety of microorganisms and conditions of bioassay than any such study previously reported.

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† Member, Subcommittee on Microbiology, Screening Panel, Cancer Chemotherapy Naional Service Center, National Cancer Institute, Public Health Service, Bethesda, Md.

A series of 200 compounds from the files of the CCNSC were studied with respect to their inhibitory activity in these 16 microbiological systems. These same compounds also were examined for cytotoxic activity in human cell cultures of normal and neoplastic origin, and the results of these studies are reported elsewhere.[4] These 200 test compounds were not selected at random from the files, but included a number of compounds known to inhibit experimental tumors *in vivo*, and others not known to have antitumor properties but known to be active (or toxic) in other biological systems.

Aside from their bearing on the screening of potential antitumor agents per se these studies also emphasize: (1) the variability in response of different microorganisms to individual inhibitory compounds, and (2) the extent to which results with a single microorganism will differ when the test medium and/or experimental conditions are varied. The latter point has been investigated extensively by determining the effects of the addition of a variety of supplements such as vitamins, amino acids, inorganic salts, and natural products on the inhibitory activity of the test compounds. The detailed results of these studies will be presented elsewhere.

Methods

Bioassay Systems Used

The microbiological systems used in each of the cooperating laboratories for these studies are listed in TABLE 1, together with notations as to time and temperature of incubation and appropriate references to the specific media employed in each bioassay system. Extensive consideration of the techniques concerned with each of these bioassay systems cannot be made here, but the methodologies used were those recommended by the American Association of Vitamin Chemists.[26] It should be noted that each of the microbiological systems used (except Test System No. 11, TABLE 1, where media were optimal) was designed originally for the bioassay of a specific growth factor; thus, the media become complete, although not necessarily optimal, upon addition of the limiting growth factor. The respective limiting growth factors were added to assay systems 1 to 7 in the minimum concentration that supported maximal growth under the prevailing experimental conditions and in excess in assay systems 8 to 10.

The method of preparation of inocula varied in detail for the different bioassay systems, but conformed to the usual standard methods.[26] In each instance the inocula used in the respective system were those that yield optimum growth in the complete test medium, and no growth in the same medium lacking one or more growth factors. Appropriate medium controls and medium blanks for each individual system were included in all titrations.

TABLE 1

BIOASSAY MICROORGANISMS AND ASSAY MEDIA USED IN ASSESSING INHIBITORY PROPERTIES OF TEST COMPOUNDS

Cooperating laboratory	Test system No.	Bioassay microorganism	Strain No.	Incubation		Test volume* (ml.)	Assay medium
				Hr.	°C.		
Foley, McCarthy, and Binns	1	*Streptococcus faecalis*	8043†	24	37	10	Folic acid[10]
Foley, McCarthy, and Binns	2	*Lactobacillus casei*	7469†	48	37	10	Riboflavin[11]
Foley, McCarthy, and Binns	3	*Escherichia coli*	6521†	48	37	10	Inorganic salts, glucose[12]
Foley, McCarthy, and Binns	4	*Lactobacillus arabinosus*	8014†	48	37	10	Nicotinic acid[13]
Foley, McCarthy, and Binns	4a	*Lactobacillus arabinosus*	8014†	48	37	10	Pantothenic acid[14]
Foley, McCarthy, and Binns	5	*Leuconostoc citrovorum*	8084†‡	48	37	10	Citrovorum factor[15]
Foley, McCarthy, and Binns	6	*Lactobacillus fermenti*	9338†	48	37	10	Thiamine[16]
Foley, McCarthy, and Binns	7	*Candida albicans*	CCRF 60112[6]	48	37	10	Inorganic salts, glucose[17]
Snell and Guirard	8§	*Saccharomyces carlsbergensis*	4228†	22	30	6	Pantothenic acid plus pantothenate[18]
Snell and Guirard	9	*Streptococcus faecalis*	8043†	14	37	6	Hydrolyzed casein, glucose, salts, vitamins[19]
Snell and Guirard	10	*Lactobacillus casei*	7469†	22	37	6	Hydrolyzed casein, glucose, salts, vitamins[20]
Snell and Guirard	3a	*Escherichia coli*	9637†	17	37	6	Inorganic salts, glucose[21]
Kidder and Dewey	11	*Tetrahymena pyriformis*	W[7]	96	25	4	Amino acids, glucose, salts, vitamins[22]
Kidder and Dewey	11a	*Glaucoma scintillans*	A[7]	96	25	4	Casein, amino acids, vitamins, salts[23]
Kidder and Dewey	11b	*Colpidium campylum*	C[7]	96	25	4	Casein, amino acids, vitamins, salts[23]
Thayer	12	*Neurospora crassa*	Wild type† 4A	48	32	20	Salts, sucrose, biotin[24]
Eagle and Foley	t	Human tissue cell cultures	Liver[8] KB[9]	144–192	37	2–3	(See Eagle and Foley[4, 25])

* Growth was determined turbidimetrically at 660 mμ in Test Systems Nos. 1 through 11b; by dry weight of mycelium in Test System No. 12; and by total protein in Test System t.

† American Type Culture Collection.

‡ *Pediococcus cerevisiae.*[5]

§ Shake cultures; all other systems are static cultures.

Detailed descriptions of each procedure may be found in the reports submitted to the CCNSC by the cooperating laboratories.[27-30]

There is some duplication among the bacterial strains used in the different laboratories. However, even though the same bacterial strain was employed, the corresponding test media differed from one another in that each was designed originally for a different purpose. For example, *Streptococcus faecalis* No. 8043* was used in a medium designed for the bioassay of pteroylglutamic acid in the presence of excess concentrations of purines and pyrimidines (Test System No. 1, TABLE 1), as well as in a medium that contained an excess of folic acid and no purines or pyrimidines (Test System No. 9, TABLE 1). Similarly, *Lactobacillus casei* No. 7469* was used in media designed for the bioassay of riboflavin (Test System No. 2, TABLE 1) and pyridoxal (Test System No. 10, TABLE 1). The inhibitory responses obtained indicated that each of these four bioassay systems were sufficiently different to warrant individual consideration in the present analyses.

On the other hand, the inhibitory responses obtained with *Lactobacillus arabinosus* No. 8014* in substrates designed for the bioassay of pantothenic (Test System No. 4, TABLE 1) and nicotinic acids (Test System No. 4a) were essentially identical. Similarly, the data derived from the use of two strains of *Escherichia coli* in similar synthetic media (Test Systems Nos. 3 and 3a, TABLE 1) were essentially identical, as were the individual data obtained with *Tetrahymena pyriformis*, *Glaucoma scintillans*, and *Colpidium campylum* (Test Systems Nos. 11, 11a, and 11b, respectively, TABLE 1). Accordingly, the individual data obtained with Test Systems Nos. 3a, 4a, 11a, and 11b (TABLE 1) have been omitted from the present analyses. The data obtained on the inhibitory response of adenineless, lysineless, and nicotinamideless mutants of *Neurospora crassa* to a representative series of test compounds were identical with those obtained with Test System No. 12 (TABLE 1) and were therefore omitted from the present analyses.

Compounds Tested

The compounds tested are listed in TABLE 2. One of the compounds (CCNSC X No. 2) could not be tested in microbiological systems because of its insolubility; 1 compound was duplicated, and 18 compounds were considered at the time of analysis to have been inadequately tested with respect to antitumor activity in that they had been tested against only 1 or 2 experimental tumors. These 20 compounds have been omitted from the present analyses.

Preparation and Titration of Test Compounds

Whenever solubility permitted, the test compound was dissolved in glass-distilled water, neutralized with NaOH or HCl, as necessary, and diluted to appropriate concentration. In some instances, to effect solution it was necessary to heat, add additional HCl, NaOH, or ethanol, or apply a combination of these treatments. Such treatment was resorted to only where necessary, and then with due regard to the stability characteristics of the compound in

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* American Type Culture Collection number.

TABLE 2

COMPOUNDS SUBMITTED FOR MICROBIOLOGICAL AND TISSUE CULTURE SCREENING

CCNSC X No.	Chemical name	NSC No.
1	*p*-Nitrobenzamide	2902
2	Ricin	
3	Tri-*n*-butyl phosphite	2675
4	Allyl lactate	1256
5	Puromycin aminonucleoside [6-dimethylamino-9-(3′-amino-3′-deoxy-β-D-ribofuranosyl)purine]	3056
6	6-Methoxyquinoline-4-aldehyde	449
7	Guanylurea sulfate	3123
8	Methionine sulfoxide γ-methylsulfinyl-α-aminobutyric acid)	3084
9	Ethanol, 2-(2-methoxyethoxy)-	2261
10	β-Mercaptopropionic acid	437
11	*p*-Anisaldehyde thiosemicarbazone	712
12	4-Acetylmorpholine	2764
13	Potassium arsenite	3060
14	10-(β-Hydroxyethyl)-7,8-dimethylpyrimido [4,5-*b*] quinoxaline-2,4-(3H,10H)dione acetate [6,7-dimethyl-9-(2′-hydroxyethyl)isoalloxazine acetate]	3064
15	2-(2,4-Dichlorophenoxy)ethanol	423
16	4-(*p*-Diethylaminostyryl)-quinoline	4240
17	2,6-Diaminopyridine	1921
18	Isoriboflavin	3100
19	Benzenesulfonhydrazide	643
20	Formamide	748
21	DL-Desthiobiotin	3085
22	2-Aminopyrimidine	1912
23	9-(γ-Di-*n*-butylaminopropylamino)-1,2,3,4-tetrahydroacridine phosphate	280
24	Deoxypyridoxine·HCl (2,4-dimethyl-3-hydroxy-5-hydroxymethylpyridine)	3063
25	Colchicine	757
26	2-Amino-1,3,4-thiadiazole	4728
27	6-Thioguanine	752
28	2,4-Dimethyl-3-pentanone semicarbazone	3121
29	Camphoric acid	824
30	7-Chloro-4-(4-diethylamino-1-methylbutylamino)-3,6-dimethylquinoline diphosphate dihydrate	355
31	Allyl *p*-chlorophenylcarbonate	2657
32	γ-Dinitrophenol (2,4-dinitrophenol)	1532
33	Di-*N*-nitrosopiperazine	339
34	1,5-Diaminobiuret	3095
35	α-Chloroacetanilide	311
36	2-(*p*-Dimethylaminostyryl)-quinoline methiodide	4238
37	Lauric acid hydrazide	1136
38	5-Pyrimidinecarboxylic acid, 1,2,3,4-tetrahydro-2,4-dioxomonohydrate	1589
39	Galactoflavin	3099
40	4,6-Diamino-1-(4′-chlorophenyl)-2,2-dimethyl-1,2-dihydro-*s*-triazine hydrochloride	3074
41	Ethyl carbamate	746
42	*p*-Biguanidobenzamide	5201
43	Dodecyl 4-*n*-nonylpyridinium chloride	2012
44	*N*-Methylacetamide	747
45	*N*-2-Hydroxyethylphthalimide	2773
46	Actidione	185
47	6-Chloropurine	744
48	4,6-Diamino-1-(3′-chlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride	3080
49	4,6-Diamino-1-(3′-bromophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride	3082
50	Diethyl ethane phosphonate	2671
51	β,β′-Dithiocyanodiethyl ether	1195
52	1,2,3,6-Tetrahydro-3,6-dioxo-2-phenyl-4-pyridazineacetic acid	3141
53	5,7-Dihydroxy-(3,1,2)oxadiazolo(*d*)pyrimidine	3098
54	1,3-Diethylurea	429
55	*n*-Lauryl thiocyanate	1481
56	Brucine sulfate	
57	4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-(2,6-xylyl)-*s*-triazine·HCl	5205
58	8-Hydroxyquinoline	2039
59	*n*-Butyl thiocyanate	1478
60	2-Amino-5-nitrothiazole	4
61	4,6-Diamino-1-(*p*-carboxyphenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride	3075
62	1-Propanol, 3-(methylthio)-	2859
63	Ethylene dithiocyanate	1911
64	Strophanthin K	4320
65	*p*-Acetylaminobenzaldehyde semicarbazone	1590
66	Netropsin hydrochloride	3067

TABLE 2—*Continued*

CCNSC X No.	Chemical name	NSC No.
67	Di-*n*-octylamine	1765
68	AA-223 Lederle	5202
69	4,6-Diamino-1-(3′,4′-dichlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride	3077
70	β-Diethylaminoethyl chloride hydrochloride	2059
71	Guanidineacetic acid (glycocyamine)	1901
72	Methyl bis(β-chloroethyl)-amine hydrochloride	762
73	Azaserine (*O*-diazoacetyl-L-serine)	742
74	10-(β-Hydroxyethyl)-7,8-dimethylpyrimido[4,5-*b*]-quinoxaline-2,4(3H,10H)-dione succinate [6,7-dimethyl-9-(2′-hydroxyethyl)-isoalloxazine succinate]	3066
75	2-Benzoyl-1-(α-pyridyl)propanol-1	198
76	4-(*p*-Diethylaminostyryl)-quinoline dihydrochloride	4242
77	2,6-Dimethyl-4-(2-oxo-2-phenylethylmercapto)pyrylium bromide	2515
78	Chloromethyl *p*-chlorophenyl sulfone	3212
79	2,6-Diaminopurine hydrate	743
80	α,α-Dimethylbenzyltrimethylammonium iodide	350
81	Cacotheline	5069
82	Narcotine	
83	10-(β-Hydroxyethyl)-7,8-dimethylpyrimido[4,5-*b*]-quinoxaline-2,4-(3H,10H)-dione [6,7-dimethyl-9-(2′-hydroxyethyl)isoalloxazine]	3065
84	2-Dimethylaminoethanol	2652
85	Meconic acid (3-hydroxy-4-oxo-4-H-pyran-2,6-dicarboxylic acid)	805
86	Salicylamide	3115
87	4-(*p*-Dimethylaminostyryl)-quinoline	4236
88	Quinidine sulfate	
89	Chloramphenicol, Chloromycetin (D-*threo*-*N*-dichloroacetyl-1-*p*-nitrophenyl-2-amino-1,3-propanediol)	3069
90	5-Iodosalicylic acid	2789
91	4,6-Diamino-1-(3′-chlorophenyl)-1,2-dihydro-2-*n*-hexyl-*s*-triazine hydrochloride	3083
92	Sodium azide	3072
93	Caffeine	5036
94	2,4′-Sulfonyldiphenol	2432
95	Folic acid (pteroylglutamic acid, PGA)	3073
96	Theobromine	5039
97	Actinomycin D	3053
98	4-Chlorochalcone	2643
99	2,4-Diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine (Daraprim, pyrimethamine)	3061
100	Methyl chloroacetate	2635
101	6-Nitrobenzimidazole	3068
102	4-(*p*-Diethylaminostyryl)-quinoline hydrochloride	4241
103	Pyronin B	3093
104	2-Amino-2-methyl-1-propanol	441
105	6-Mercaptopurine hydrate	755
106	Santonin	4900
107	1,9-Dimethanesulfonoxynonane	3052
108	Methyl carbamate	3054
109	3,3′-Diamino-4,4′-dihydroxyarsenobenzene dihydrochloride	3097
110	Pyrogallol	5035
111	8-Azaxanthine	756
112	Methyl green	3091
113	Isopropyl *N*-phenylcarbamate	2105
114	DL-Ethionine	751
115	Theophylline	2066
116	Berberine sulfate	
117	Picryl chloride	1872
118	Chloral hydrate	3210
119	1-Phenyl-3,3-dimethyltriazene	3094
120	4,6-Diamino-1-(4′-chlorophenyl)-2-*n*-hexyl-1,2-dihydro-*s*-triazine hydrochloride	3081
121	Ethylhydrocupreine hydrochloride	
122	Aminopterin (4-aminopteroylglutamic acid)	739
123	Strychnine	
124	α-Cyano-β-phenylacrylic acid	298
125	Dimethyl sulfoxide	763
126	4-Amino-6-(*p*-chloroanilino)-1,2-dihydro-2,2-dimethyl-*s*-triazine$\cdot 1H_2O$	5203
127	2-Allylamino-5-methyl-1,3,4-thiadiazole hydrochloride	4731
128	Purine	753
129	2-Naphthoic acid, 3-hydroxy-7-sulfo-	2852

TABLE 2—*Continued*

CCNSC X No.	Chemical name	NSC No.
130	Methyl 3-pyridyl ketone (3-acetyl pyridine)	761
131	Phenol, *p*-chloro-	2877
132	D-Glucosamine hydrochloride	758
133	A-Methopterin	740
134	2-Acetamido-1,3,4-thiadiazole	4729
135	4-(*p*-Dimethylaminostyryl)-quinoline methiodide	4239
136	Codeine sulfate·$5H_2O$	
137	Cyclopropanecarboxylic acid	1112
138	Methylene blue chloride (methylthionine chloride)	3089
139	8-Aza-2,6-diaminopurine sulfate	3057
140	Cotarnine hydrochloride	
141	β-Benzoylpropionic acid	2092
142	3,5-Dibromoanthranilic acid	886
143	4-Amino-6-anilino-1,2-dihydro-2,2-dimethyl-*s*-triazine	5204
144	*N*-Phenylsuccinimide	2359
145	Thebaine hydrochloride ethanolate	
146	Lauranilide	3181
147	Trichloroacrylic acid, sodium salt	2639
148	Tetrahydrothebaine hydrochloride trihydrate	
149	Myleran (1,4-dimethanesulfonyloxybutane)	750
150	Resorcinol	
151	β-2-Thienylalanine	754
152	Benzylidene adonitol (or dibenzylidene adonitol) (1,3:2,4-di-*O*-benzylidene-DL-ribitol)	1355
153	Deacetyl-*N*-methylcolchicine	3096
154	Protopine hydrochloride	
155	2,4-Diamino-6-hydroxy-5-phenylazopyrimidine	5200
156	*p*-Fluoroaniline	579
157	*N*-(2-Carboxyethyl)-L-tyrosine	3143
158	Hydrocortisone	741
159	2,2′-Sulfinyldiethanol	2897
160	β-Chloropropionic acid	2183
161	Benzimidazole	759
162	8-Azaguanine	749
163	Benzenephosphonic acid	145
164	3-Pyridine sulfonic acid, sodium salt	1578
165	2-(1,1-Dimethyl-3-guanidino)-4-methyl-6-chloroquinazoline nitrate hydrate	1228
166	4,6-Diamino-1-(*m*-bromophenyl)-1,2-dihydro-2-(*n*-undecyl)-*s*-triazine·HCl	5206
167	Benzoic acid, *m*-sulfo-	2853
168	Acetylene dicarboxylic acid	1903
169	4-(*p*-Dimethylaminostyryl)-quinoline methiodide	4239
170	2,4-Diamino-5-(3′,4′-dichlorophenyl)-6-ethylpyrimidine	3062
171	Quinine hydrobromide	
172	5-Nitro-2-furfurylidene semicarbazone (nitrofurazone)	2100
173	*n*-Heptylamine	2074
174	Neutral red (toluylene red)	3092
175	Tetrapropyl thiopyrophosphate	2550
176	3-Methoxypropylamine	552
177	Morphine sulfate·$5H_2O$	
178	2-Methylpseudourea hydrochloride	1896
179	Ethylene sulfite (cyclic ester)	3225
180	Phenol, 2,4-dichloro-	2879
181	*p*-Acetamino-*m*-anisidine	1873
182	*S*-Benzylthiuronium chloride	3206
183	Apomorphine hydrochloride	
184	Thiopanic acid (pantoyltaurine)	3086
185	Puromycin dihydrochloride [6-dimethylamino-9-(3′-*p*-methoxy-L-phenylalanylamino-3′-deoxy-D-ribosyl)-purine·2HCl]	3055
186	5,6-Diaminouracil, hemisulfate	3102
187	4-Amino-6-anilino-1,2-dihydro-2-phenyl-*s*-triazine	3076
188	*N*-Methylformamide	3051
189	*N*-(5-Nitro-2-furfurylidene)-1-aminohydantoin (Furadantin)	2107
190	Nicotine	5065
191	2-Hydroxy-4,6-dimethyl pyrimidine hydrochloride	2030
192	Cinchonidine	
193	Crystal violet (gentian violet)	3090
194	Benzenesulfonic acid, diester with diethylene glycol	2857
195	Methylolacrylamide	553
196	4-(*p*-Dimethylaminostyryl)-quinoline methochloride	4237
197	2-Ethylamino-1,3,4-thiadiazole hydrochloride	4730
198	Benzotriazole	3058
199	5-Chlorobenzimidazole	3059
200	Oxophenarsine·HCl (2-amino-4-arsenosophenol hydrochloride)	3087

question. In those instances where ethanol (or, in rare instances, other organic solvents) were used, appropriate solvent dilutions were assayed for inhibitory activity. One of the test media (Test System No. 11, TABLE 1) contained Tween 80, which aided in dissolving certain of the test compounds. The stock solutions so prepared were sterilized by filtration through ultrafine, sintered glass (Morton) filters in two laboratories (Snell and Guirard, and Kidder and Dewey, TABLE 1), while in the other two laboratories (TABLE 1), the test compound was assumed to be sterile and, accordingly, was handled aseptically as described above, including the use of sterile glass-distilled water.

In either case, appropriate concentrations of test compound and the specific growth factor (for example, pteroylglutamic acid in Test System No. 1, TABLE 1) required by each bioassay system were added aseptically to the appropriately diluted and previously autoclaved media in such a way that the total volume in each culture tube was that indicated in TABLE 1 for each bioassay system. All compounds were first tested in serial tenfold dilutions resulting in final concentrations of 0.1 to 1000 (or more) gamma/ml. of bioassay media. The results of this preliminary titration permitted a second test that defined the inhibitory concentration (or range of concentration) more closely. After inoculation with appropriately prepared and diluted inocula, the titrations were incubated as indicated for the individual bioassay systems in TABLE 1. Following the required period of incubation, growth was determined turbidimetrically in a colorimeter adjusted so that the respective medium blanks read zero at 660 mμ in bioassay systems Nos. 1 through 11 (TABLE 1). Growth was determined by the dry weight of the mycelia in *Neurospora crassa* bioassays (Test System No. 12, TABLE 1).

Several of the compounds tested were highly pigmented and therefore produced color in the bioassay media that interfered with turbidimetric determinations. In most instances this interference could be eliminated by titration of the compound in uninoculated bioassay media, using the resulting colored blanks to adjust the colorimeter to zero at 660 mμ. However, even this procedure was of little use with a few of the pigmented compounds and, in these instances, the range of concentrations inhibiting growth could be estimated only by visual inspection.

The concentration of test compound that allowed only half-maximal growth (50 per cent minimal inhibiting dose, or ID_{50})[31] was interpolated from a graphic plot of titration data so obtained, using as maximal growth those values obtained in the respective specific growth factor controls included in the same titration, as illustrated schematically in FIGURE 1; thus, the ID_{50} has been expressed as the concentration in gm./ml. that reduces growth to one half of that obtained in the absence of the test compound. Although the ID_{50} was determined with considerable precision (for example, FIGURE 2), its absolute value may vary somewhat with the exact conditions of bioassay. For this and other reasons, the ID_{50} values have been expressed as a range that brackets the exact 50 per cent inhibitory concentration in the present analyses.

Using these data as reference, the ID_{50} in microbiological assay systems that classified as "positive" the maximum number of compounds with reported experimental antitumor activity *in vivo* was chosen as the arbitrary differential

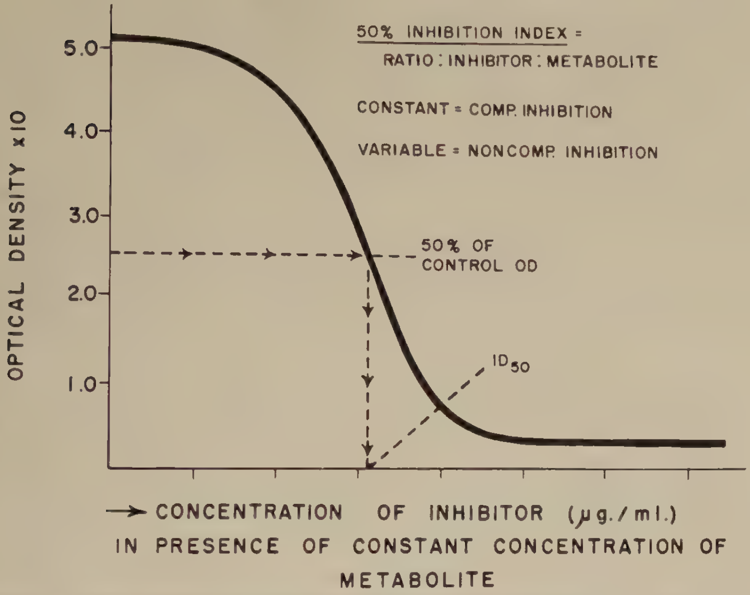

FIGURE 1. Determination of ID_{50} of test compounds in microbiological bioassay systems.

criterion for classification of a compound as "positive" or "negative." It is of interest to note that each investigator, upon independent analysis of his own data, chose the same differential: inhibitory activity at less than 1.0×10^{-3} gm./ml. It was immediately apparent also that, since none of the individual microbiological systems detected all of the compounds reported to be "active" against experimental tumors, the criterion for positivity in the present analyses should be an ID_{50} at less than 1.0×10^{-3} gm./ml. in any one (or more) of the bioassay systems used.

Criteria for Classification of Test Compounds as "Tumor Active"

It is obvious immediately that the reliability of any screening procedure depends in large part upon the more or less arbitrary criteria selected to adjudge "activity." In the present analyses the only available point of reference by which to adjudge the significance of positivity in a microbiological assay is the reported activity of the test compound against experimental tumors *in vivo*. The evaluation of the antitumor activity of these two hundred compounds was identical with that used as the point of reference in the human cell culture studies with the same compounds,[4] and was obtained from three sources:

(1) Published data on the activity of the various compounds in experimental tumor systems. The original investigator's evaluation of the compound as "active" or "inactive" generally was used in the present analysis. These data are summarized in a report from the CCNSC.[32] In a few instances the criteria for activity differed so widely from those set up by the CCNSC as to require either re-evaluation of the data, or a retest.

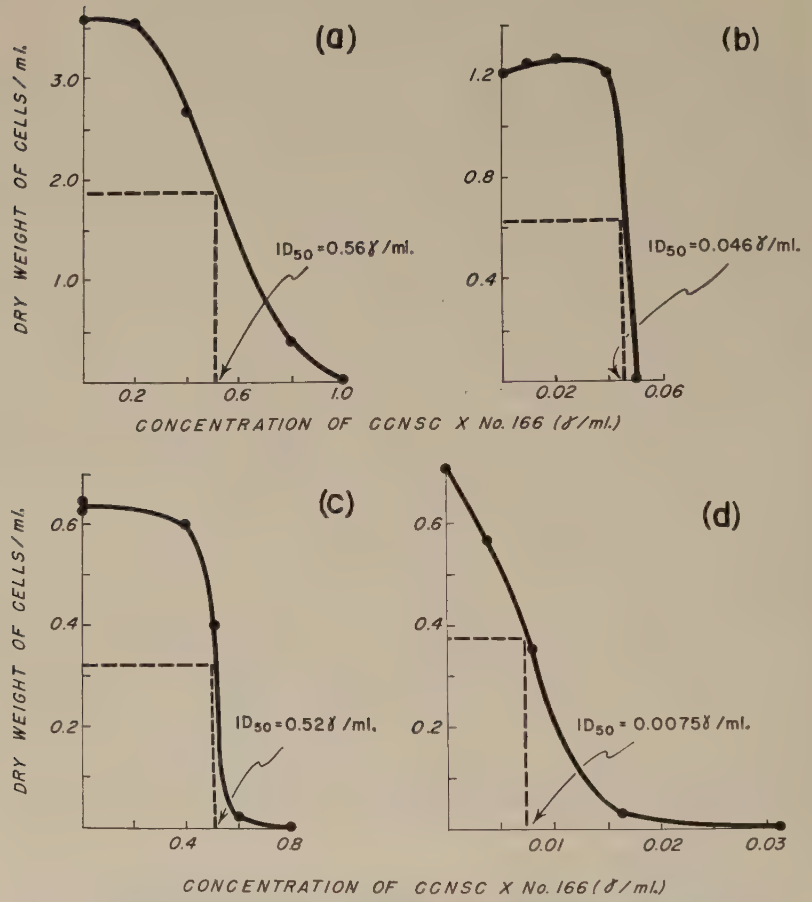

FIGURE 2. Determination of ID$_{50}$ of inhibitory compound in four microbiological bioassay systems: (a) *Saccharomyces carlsbergensis* (Test System No. 8); (b) *Escherichia coli* (Test System No. 3a); (c) *Lactobacillus casei* (Test System No. 10); and (d) *Streptococcus faecalis* (Test System No. 9). Optical density has been converted to dry weight of cells from appropriate calibration curves.

(2) A large body of data generously made available from the files of the CCNSC,[32] detailing activity *in vivo* against Sarcoma 180 tumors in Swiss albino mice, Ca 755 mammary adenocarcinoma, and L 1210 lymphatic leukemia in C57/DBA hybrids. The criteria set up by the center were used in the evaluation of these results as "active" or "negative."

(3) Supplementary tests against P 1534 lymphatic leukemia in DBA/2 mice, C 1498 myelogenous leukemia in C57BL/6 mice, and DBRB adenocarcinoma in DBA/1 mice; all carried out at The Children's Cancer Research Foundation, Boston, Mass., under the auspices of Sidney Farber.

Only those compounds tested against at least three experimental tumors were included for analysis. Compounds were adjudged arbitrarily as "tumor-active, ++" (if they had been reported to have a definite inhibitory effect against at least two different experimental tumors) and "tumor-active, +" (if they had been reported to affect only one experimental tumor). Compounds giving a positive and a "borderline" result in two different tumor systems were arbitrarily adjudged "tumor-active, ++"; compounds with borderline activity in two tumor systems were adjudged "tumor-active, +." As pointed out previously,[4] some necessary reservations must be recognized with respect to use of experimental tumor results as a base of reference for comparison with either cell cytotoxicity or antimicrobial activity. These reservations will be considered in the discussion of the present results.

RESULTS

The inhibitory activity observed for each of 198 compounds in each of 12 microbiological assay systems is tabulated in TABLE 3. The cytotoxicity of these compounds in tissue cell cultures,[4] also has been recorded in TABLE 3 for purposes of other comparisons.[33] Data on the 18 compounds that, at the time of analysis, were considered to have been inadequately tested with respect to antitumor activity also are included in TABLE 3, so that, in the event that further evaluation against experimental tumors is made, the *in vitro* activity exhibited by these compounds will be available for comparison. However, it appears from consideration of all possible results that may be obtained with these 18 compounds in additional experimental tumor systems that omission (or addition) of these data does not materially affect the conclusions drawn from the present analyses. It should be mentioned that the classification of compounds as "tumor ++," "tumor +," and "tumor −" in TABLE 3 is based upon extensive surveys of antitumor activity as reported in the literature.[32] Detailed comparisons with antitumor activity as adjudged by the CCNSC Mouse Tumor Screening Program will be made elsewhere in this monograph.[33]

Inspection of TABLE 3 indicates that, in general, there is little direct correlation between the results obtained in different microbiological systems. The same compound may exhibit an extreme range of activity in different test systems; for example, CCNSC X No. 25 (colchicine) was inactive at 1.0×10^{-3} gm./ml. in all 12 microbiological systems, yet was extremely active at less than 1.0×10^{-8} gm./ml. in tissue cell cultures.[4] Other compounds, for example, CCNSC X No. 200 (2-amino-4-arsenophenol hydrochloride) were active only at $1.0 \times 10^{-3} - 1.0 \times 10^{-4}$ in one system, while exhibiting essentially similar activity ranging from less than 1.0×10^{-4} to 1.0×10^{-6} gm./ml. in 11 of the 12 microbiological systems. Highly active compounds that were inhibitory in all 12 microbiological systems also exhibited a variable ID_{50} in different test systems; for example, CCNSC X No. 97 (actinomycin D) was inhibitory in all 12 microbiological systems at 1.0×10^{-4} gm./ml. or less, with an ID_{50} between $1.0 \times 10^{-6} - 1.0 \times 10^{-7}$ in two, between $1.0 \times 10^{-7} - 1.0 \times 10^{-8}$ in four, and less than 1.0×10^{-8} gm./ml. in two individual test systems.

TABLE 3
ACTIVITY OF TEST COMPOUNDS ON GROWTH OF CERTAIN MICROORGANISMS

CCNSC X No.	ID$_{50}$*					
	<3	3–4	4–5	5–6	6–7	7–8 or greater†
Tumor ++						
5	1–7, 10–12	8, 9		t		
13	5	4, 6–8, 12	1–3, 9, 10	11	t	
14	3–7, 12	1, 8, 11		9, 10, t	2	
15		1–7, 9–12	8	t		
16		1–7, 9–11		8, 12	t	
18	12, t	8–11	1–7			
20	1–12, t					
24	1, 3–7, 9–12	2	8	t		
25	1–12					t†
26	1–7, 10–12	8, 9		t		
27	3, 7, 12	1, 2, 5, 6, 8, 11	4	10	t	9
30	1, 2, 5–9	3, 4, 10–12		t		
34	5, 6	1, 2, 4, 7, 9	3, 8, 11, 12, t	10		
39	12, t	1, 3–11			2	
40	2, 3, 7, 12		4, 5, 8, 10, 11		t	1, 6, 9
41	1, 3–9, 11, 12, t		2	10		
44	1–12, t					
46	1–7, 9, 10			11	t	8, 12
47	1, 4, 5, 7–9, 12	2, 6, 10, 11	3, t			
48	7	2–4, 6, 12	5, 8, 10	11	t	1, 9
49	7	2, 3, 4, 6, 12	5, 8	10, 11	1, t	9
57	1–7, 8, 10–12	9		t		
64	1–12					t
66			5, 7, 8, t	1–4, 6, 9–12		
68	3, 7, t		2, 4, 5, 8–11	1, 6	12	
69	7	2, 3, 6, 12	5, 8, 10	11	4	1, t 9,
70	1–7, 12	8–11		t		
72	1, 9	2–7	10	11, 12	t	8
73	4, 5	2, 7, 10, 11	1, 6, 9	3, 8, 12	t	
74	4–10, 12	1, 3, 11		2, t		
76		1–7, 9–11		8	12, t	
79	4	1, 2, 5, 6, 8, 11	3, 7, 9	10, 12, t		
83	3, 5, 7, 12	4, 6, 8–11	1	t	2	
87	2–6	1, 7, 10	8, 9, 11, 12			t
91	7	3, 11	2, 8, 10, 12		4, 5, 6, t	1, 9†
92		4, 5, 6, 9	1, 2, 10	3, 7, 11, t	8, 12	
97			3, 7, 8	11	5, 12	1, 2, 4, 6,† 9,† 10, t
99	7	3, 12	8, 10	5, 11	t	1, 2, 4, 6, 9
102	1–5, 7	9, 10, 11		6, 8, 12	t	
105	7, 12	2, 3, 5, 6, 8, 9, 11	1, 4	10	t	
107	4, 5, 6	2, 7, 9, 10, 12	1, 3, 8, 11	t		

* Negative logarithm of concentration range within which there was 50 per cent inhibition of growth.

† These compounds were inhibitory at concentrations less than 10^{-8} gm./ml. (that is, negative log > 8).

TABLE 3—*Continued*

CCNSC X No.	ID$_{50}$*					
	<3	3–4	4–5	5–6	6–7	7–8 or greater†
109	7	12	3, 8, 11	1, 2, 4–6, 9, 10, t		
113	t	5, 8–10, 12	1–4, 6, 7, 11			
114	1–7, 9, 10, t	11		12	8	
119		2, 5, 9–11	1, 3, 4, 6–8, 12, t			
120	7	3, 8, 11, 12	2, 4, 5, 6, 10	t	1	9
122	7, 11	3		12	5, 8	1, 2, 4, 6, 9,† 10,† t
128	1, 4, 5, 6, 11, 12	2, 3, 7, 8	9	10	t	
133	7	3		5, 8, 12	11	1,† 2,† 4,† 6,† 9,† 10,† t
134	4–7, 9–12, t	1, 2, 8	3			
135		7	1, 3, 6, 8, 9, 11	2, 4, 5, 10, 12	t	
138		8	9	1–7, 10–12	t	
143	1–8, t	10, 12	9, 11			
149	1–7, 12, t	8, 9, 11	10			
153	7, 9, 10, 12	2, 5, 6, 8, 11	1, 3, 4			t†
158	1, 3, 5, 6, 12, t	4, 7, 9	2, 8, 10	11		
162	3, 7	1	2, 4, 5, 6, 8	11	12, t	9, 10†
166	7	3	11	12, t	5, 6, 8, 10	1,† 2, 4, 9†
170	7	12	2, 3, 8	5, 10, 11	6, t	1, 4, 9†
171	6–9	1–5, 10–12	t			
174		9	1–7, 10–12	8, t		
185	12	7, 8	2, 3, 5	1, 4, 6, 9, 10	11, t	
187	4, 7	2, 3, 5, 8–10, 11, 12	1, 6		t	
188	1–12, t					
193			11		1–7, 9, 10, 12	8, t
196			5, 7, 8, 11	1–4, 6, 9, 12	10	t
197	1–11, t	12				
200		7	1, 3, 4, 6, 8, 11, 12	2, 5, 9, 10, t		
Tumor +						
19	5–7	1–4, 8, 10, 11	9, 12, t			
21	1, 3–9, 11, 12, t	2, 10				
29	7	1–6, 8–12		t		
32		1–3, 5–7	4, 8–10, 11, t	12		
36			3, 5, 8, 9, 11	1, 2, 4, 6, 7, 10, 12		t
51	2–6	1, 7, 9, 10	3, 8, 11, 12	t		
56	1, 3–10, 12	2, 11		t		
58			2, 6	1, 3–5, 7, 9	8, 10, 11, t	12†

* Negative logarithm of concentration range within which there was 50 per cent inhibition of growth.

† These compounds were inhibitory at concentrations less than 10^{-8} gm./ml. (that is, negative log > 8).

TABLE 3—*Continued*

CCNSC X No.	ID50*					
	<3	3–4	4–5	5–6	6–7	7–8 or greater†
63		2	1, 3, 4, 6, 7, 9, 10, 12	5, 8, 11, t		
95	2, 3, 5–11, t	1, 4, 12				
110	1, 2, 4–7, 9, 10	3	8, 11, 12, t			
112		11	1–7, 9, 10, t	8, 12		
150	1, 2, 4–10, t	3, 11, 12				
151	1, 2, 5–7, 9, 10, t	3, 4, 11	12	8		
154	1, 2, 5–8, 12	3, 4, 9, 10		11, t		
165	7	1–6, 8, 9, 12	10, 11		t	
172	12	4, 7, 8	2, 6, 10, 11	1, 3, 5, 9, t		
182	3, 5–7, 9, 12	1, 4, 8, 10, 11	2		t	
186	12, t	1, 3, 6–11	2, 4, 5			
198	1, 4, 5, 10, t	2, 3, 6–9, 11, 12				
199	7	1–6, 9–12	8, t			
Tumor —						
1		2, 4, 9	1, 3, 5–7, 10–12	8		
3		8, 10–12, t	1–7, 9			
4	1, 2, 4–8, 10–12, t	3, 9				
6		1, 2, 4–7	3, 9–12, t	8		
7	1–7, 9–12	8, t				
8	2–12	t	1			
9	1–12	t				
10	2, 4–7, 9–11,	1, 3, 8, 12, t				
11	12	1, 2, 4–6	3, 7–10	11, t		
12	8, 10–12	2, 9, t	1, 3–7			
17	12	1–11, t				
22	1–10, 12	11, t				
31	1, 4, 6, 7	3, 5, 10, 11, t	2, 8, 9, 12			
35	2, 5	4, 6, 9, 10	1, 3, 7, 8, 11	12, t		
37	1–3	t	4–7, 9, 11, 12	8, 10		
38	1–7, 11, 12	8–10, t				
42	1, 2, 4–8, 10, 12	3, 9, 11, t				
45	1–7, 11, 12	8–10, t				
50	1–12	t				
52	3, 5, 7, 11, 12	8–10, t	1, 2, 4, 6			
53	7, 8, 11, 12	1–5, 10, t	6	9		
54	1–12	t				
55	1–7, 12	10, 11	8–9		t	
59		8–12, t	1–7			
60		1,2,4, 5, 7, 9, t	3, 8, 10–12	6		
61	1–8, 10–12	9, t				
62	1–7, 10–12	8, 9, t				

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* Negative logarithm of concentration range within which there was 50 per cent inhibition of growth.

† These compounds were inhibitory at concentrations less than 10^{-8} gm./ml. (that is, negative log > 8).

TABLE 3—*Continued*

CCNSC X No.	ID$_{50}$*					
	<3	3–4	4–5	5–6	6–7	7–8 or greater†
67			3, 5–7, 11	1, 2, 4, 8–10, 12, t		
71	1–12					
77	1–6, 9, 10	7	8, 11, 12, t			
78		2, 11, t	1, 3–7, 9, 10, 12	8		
80	1, 2, 4–12	3, t				
82	12	11	1–10	t		
84	1–3, 5–12	4, t				
85	1–12	t				
86	7	1–3, 5, 6, 8–12, t	4			
89	7, 8, 12		11, t	1, 2, 4–6, 10	3, 9	
93	1–7, 10	8, 9, 11, 12, t				
94	7	1, 3, 5, 6, 8, 9, 11, 12	2, 4, 10, t			
96	1–10, 12		11, t			
98	2, 12	5, 6, 9, 10	1, 3, 4, 7, 8, 11, t			
100	4, 5, 10	1, 2, 3, 7, 9	6, 8, 11, t	12		
103			3, 7, 11	1, 2, 4–6, 8, 9, 12	10, t	
104	1–3, 5–9, 11	4, 10, 12, t				
108	1–12	t				
111	8, 11	1–3, 5–7, 12	4, t		9, 10	
115	1–8, 10, 12	9, t	11			
117		t	1, 2, 4–7, 9–11	3	8, 12	
118	1–7, 9, 10	11, 12, t	8			
121	7, 8	1–6, 9, 10, 12	11, t			
123	12	1, 6, 8–10, t	2–5, 7, 11			
124	1, 3, 5, 7	2, 4, 6, 8–12, t				
125	1–11	12, t				
126	1–8	9–12	t			
127	1–7, 9, 10	8, 11, 12, t				
129	1–7, 10–12	8, 9, t				
130	1–12	t				
131		1–7, 9–11	8, 12, t			
132	1–12	t				
136	1–10, 12	11, t				
137	2–6	1, 7–12, t				
139	12	1, 2, 4–8, 11, t	3, 10		9	
140	1, 2, 4–9, 11, 12	3, 10	t			
141	2, 3, 5, 7, t	1, 4, 6, 8–12, t				
142		1–8, 10, t	9, 11, 12			
144	1–12	t				
145	1–7, 10, 12	8, 9, 11	t			
147	1–12	t				
152	12	t	1–11			

* Negative logarithm of concentration range within which there was 50 per cent inhibition of growth.

† These compounds were inhibitory at concentrations less than 10^{-8} gm./ml. (that is, negative log > 8).

TABLE 3—*Continued*

CCNSC X No.	ID$_{50}$*					
	<3	3–4	4–5	5–6	6–7	7–8 or greater†
156	1, 2, 4–7, 10,	3, 8, 9, 11, 12 t				
159	1–12	t				
161	1–7, 9	8, 10–12, t				
163	1–5, 7, 8, 10, 11	6, 9, 12, t				
164	1, 2, 4–12	3, t				
167	1, 2, 4–11	3, 12, t				
168	4–10	1–3, 12, t	11			
173		1–7, 11, t	8–10			
175	12	1–6, t	7–11			
176	1–3, 5–7, 9–12	4, 8, t				
177	1–12	t				
178	1–10, 12	11, t				
179	1–12	t				
181	1–7, 11, 12	8–10, t				
183		1–7, t	8–12			
184	1, 3, 5–12	2, 4, t				
189	12	4, 7, 8, t	10, 11	1–3, 5, 6, 9		
190	1–12	t				
191	1–12	t				
192	12	5, 8–10	1–4, 6, 7, 11, t			
194	3, 7, 10, 12	1, 2, 4–6, 9, 11, t	8			
195	1–10	11, 12, t				
Tumor incomplete‡						
23	2, 6–8	1, 3, 5, 9, 10, 12	4, 11	t		
28	5	2, 8–12, t	1, 3, 4, 6, 7			
33	1, 3, 5–9, 11, 12	2, 10, t	4			
43		11	7		1–6, 8, 12	9, 10, t
65	12	1, 2, 5, 6, 8–11, t	3, 4, 7, 12			
75	1, 4–8	2, 3, 9–12, t				
81	1–8, 12	9–11	t			
88	6, 7, 12	1, 3–5, 8–11	2, t			
90		1, 3–5, 7, 8, 10–12	2, 6, 9, t			
101	4, 5, 7	1, 3, 6, 8–12	2, t			
106	12	8–10, t	1–7, 11			
116	11	2–10, 12, t	1			
146	1, 2, 5, 6, 12	3, 9–11, t	4, 7, 8			
148	1–10, 12	11, t				
155	12	3, 7, 8, 11	2, 6, 10, t	4	1, 5, 9	
157	8–12	1, 7, t	3–6	2		
160	1, 2, 4–10	3, 11, 12, t				
180		5, 7, 11, t	1–4, 6, 8–10	12		

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* Negative logarithm of concentration range within which there was 50 per cent inhibition of growth.

† These compounds were inhibitory at concentrations less than 10^{-8} gm./ml. (that is, negative log > 8).

‡ Mouse tumor data incomplete at time of analysis.

Sensitivity of Individual Bioassay Systems in Detecting Tumor-Active Compounds

The response of each of the individual microbiological systems is correlated with the reported antitumor activity of the test compounds in TABLE 4. As was expected, no single bioassay system detected all of the compounds, and there was considerable variation between individual systems. For example, of the 89 compounds with reported experimental antitumor activity, 82 per cent were classified as positive with *Tetrahymena pyriformis* (Test System No. 11), while only 39 per cent were so classified with *Candida albicans* (Test System No. 7). Although between these extremes of sensitivity the proportion of tumor-active compounds classified as positive did not vary materially from one system to another, it must be emphasized that the same compound was not necessarily so classified by each microbiological system.

This variability in the kind of compound classified as positive, even by the same microorganism used in test media designed for different purposes, is illustrated by comparison of the response of the two *Lactobacillus casei* bioassay systems (Test Systems Nos. 2 and 10, TABLE 1). Although the number of compounds with reported experimental antitumor activity classified as positive by either test system was comparable (73 per cent and 76 per cent, respectively, TABLE 4), the compounds so classified were not identical in each system, there being 15 instances in which discrepant results were obtained among the 68 compounds classified as positive with *Lactobacillus casei* (Test System No. 10), as illustrated in TABLE 5. This particular discrepancy is due in large part to the fact that several of the test compounds were antimetabolites of purines and pyrimidines, which are effective in microbiological systems only when the purine and pyrimidine content of the test medium is minimal. The medium used in Test System No. 10 contained no added purine or pyrimidine, while that used in Test System No. 2 (designed for a different purpose) contained a yeast supplement and photolyzed peptone. The sensitivity of Test Systems Nos. 1, 4, 5, and 6 (and perhaps others) also may be decreased for the same (or similar) reasons, since purines and pyrimidines are added components in each of these test media.

It should be mentioned that microbiological assay systems can be devised readily by choice of proper strain and medium to detect compounds exhibiting antimetabolite activity against any specific essential metabolite. For example, amino acid antagonists such as β-2-thienylalanine are quite inhibitory under conditions where the corresponding metabolite (in this instance, phenylalanine) is not present or is present only in limiting concentrations, as with *Saccharomyces carlsbergensis* (Test System No. 8, TABLE 1) and *Escherichia coli* (Test System No. 3a, TABLE 1). However, this antagonist is completely inactive in media containing a concentration of phenylalanine sufficient to prevent (or reverse) inhibition, for example, with *Lactobacillus casei* and *Streptococcus faecalis* (Test Systems Nos. 10 and 9, TABLE 1) bioassay systems.

It is obvious that results obtained in any one of the individual microbiological assay systems could be varied in either direction by appropriate alterations in the composition of test medium. These facts must be borne in mind when the results obtained from several microbiological assay systems are compared

TABLE 4

CORRELATION OF RESPONSE OF INDIVIDUAL SCREENING SYSTEMS WITH ANTITUMOR ACTIVITY

Tumor status	Number of com-pounds	Screening system*																									
		1		2		3		4		5		6		7		8		9		10		11		12		t	
		(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)	(+)	(−)
Tumor++	68																										
No.		46	22	50	18	43	25	41	27	38	30	42	26	26	42	55	13	51	17	53	15	54	14	42	26	54	14
%		68	32	73	27	63	37	60	40	56	44	62	38	38	62	81	19	75	25	78	22	79	21	62	38	79	21
Tumor+	21																										
No.		14	7	15	6	17	4	15	6	10	11	11	10	9	12	16	5	14	7	15	6	19	2	15	6	16	5
%		67	33	71	29	81	19	71	29	48	52	52	48	43	57	76	24	67	33	71	29	90	10	71	29	76	24
Total positive	89																										
No.		60		65		60		56		48		53		35		71		65		68		73		57		70	
%		67		73		67		63		54		60		39		80		73		76		82		64		79	
Negative	91																										
No.		39	52	36	55	42	49	40	51	33	58	38	53	30	61	50	41	54	37	44	47	51	40	37	53	19	72
%		43	57	40	60	46	54	44	56	36	64	42	58	33	67	55	45	59	41	48	52	56	44	41	59	21	79

* See TABLE 1 for identification.

TABLE 5

COMPARISON OF RESPONSE OF *LACTOBACILLUS CASEI* TO THE SAME TEST COMPOUNDS ASSAYED IN DIFFERENT SUBSTRATES

Compound CCNSC X No.*	Classification on basis of ID_{50} of less than 1.0×10^{-3} gm./ml. in:	
	Test System No. 2†	Test System No. 10†
24	+	−
30	−	+
40	−	+
51	−	+
56	+	−
70	−	+
74	+	−
87	−	+
102	−	+
134	+	−
143	−	+
149	−	+
153	+	−
154	−	+
198	+	−

* See TABLE 2 for identification.
† See TABLE 1 and text.

with each other, or with the results obtained either in tissue cell cultures or experimental tumors *in vivo*. This variability in the response of individual bioassay systems to the same test compound is reflected in the improved results obtained by the use of combinations of microbiological systems for screening.

Incidence of "False Positives" in Individual Bioassay Systems

A "false positive," as defined here, is a compound that has been reported to have no demonstrable antitumor effect on the experimental tumors against which it happened to have been tested and was inhibitory by the arbitrary criterion used in these analyses, that is, an ID_{50} at less than 1.0×10^{-3} gm./ml. in any one of 12 microbiological systems. The frequency of these so-called false positives in the individual microbiological assay systems is recorded in TABLE 4. In general, the frequency of false positives varied with the sensitivity of the test system; those individual systems detecting the greatest percentage of tumor-active compounds also exhibited the highest rate of false positives.

It is apparent that reliability will vary with the arbitrary criteria selected to adjudge positivity. If, instead of less than 1.0×10^{-3}, for example, 1.0×10^{-4} gm./ml. or less is chosen as the differential concentration, the incidence of false positives will be reduced substantially, but only at the expense of reduced sensitivity, that is, the number of compounds with reported experimental antitumor activity that are "missed" is increased substantially, as illustrated in TABLE 6.

TABLE 6

EFFECT OF CRITERIA FOR POSITIVITY ON SENSITIVITY OF MICROBIOLOGICAL BIOASSAY SYSTEMS NOS. 1 TO 7 (INCLUDING NO. 4a)*

Tumor status	Number of compounds	Number of compounds inhibitory at (gm./ml.):			
		10^{-3} or less		10^{-4} or less	
		No.	%	No.	%
Tumor ++	68	54	79	45	66
Tumor +	21	21	100	10	46
Tumor −	91	56	61	35	42

* See TABLE 1.

Comparison of Degree of Inhibitory Activity of Tumor-Active and Tumor-Negative Compounds

Obviously it is difficult to make direct comparisons, in view of the quantitative and qualitative variability in the response of individual bioassay systems to the same test compound. However, an interesting composite comparison of the tumor-active and tumor-negative compounds can be made if a differential criterion is applied to the inhibitory activity observed in any 1 of the 12 microbiological systems. For example, if the tumor-active and tumor-negative compounds are examined for inhibitory activity in any 1 of the 12 microbiological systems at 1.0×10^{-5} gm./ml. or less, 57 per cent of the tumor-active compounds are positive at this concentration, whereas only 17 per cent of the tumor-negative compounds exhibit this degree of inhibitory activity (TABLE 7). Or, expressed in another way, of the 67 compounds in this series that were inhibitory at concentrations of 1.0×10^{-5} gm./ml. or less in any of the 12 microbiological systems, 51 (76 per cent) have been reported to be active *in vivo* with 1 or more experimental tumors.

Sensitivity of Combinations of Bioassay Systems in Detecting Tumor-Active Compounds

Eighty-four (95 per cent) of the 89 compounds in this series that were reported to exhibit experimental antitumor activity were classified as positive

TABLE 7

COMPARISON OF DEGREE OF INHIBITORY ACTIVITY IN MICROBIOLOGICAL SYSTEMS OF COMPOUNDS WITH AND WITHOUT REPORTED EXPERIMENTAL ANTITUMOR ACTIVITY

Tumor status	Number of compounds	ID_{50} at:			
		1.0×10^{-5} or less		More than 1.0×10^{-5}	
		No.	%	No.	%
Active*	89	51	57	38	43
Negative	91	16	17	75	83

* Tumor++ and tumor+ compounds.

by at least 1 of the 12 microbiological assay systems, using as the arbitrary criterion for positivity an ID$_{50}$ of less than 1.0×10^{-3} gm./ml. This was accomplished with a frequency of so-called false positives of about 60 to 65 per cent, even in this selected series, which contained a considerable number of compounds known to be highly reactive and/or toxic in various other biological systems.

However, all of these 12 microbiological systems are not required to obtain this degree of sensitivity. For example, combinations of *Tetrahymena pyriformis* (Test System No. 11), *Streptococcus faecalis* (Test System No. 9), *Lactobacillus casei* (Test System No. 2), and *Neurospora crassa* (Test System No. 12), as shown in TABLE 1, will classify as positive the same 84 (95 per cent) of the 89 compounds reported to exhibit experimental antitumor activity, as illustrated in TABLE 8. *Lactobacillus casei* (Test System No. 10) in another substrate can be substituted for *Tetrahymena pyriformis* in this combination with no loss in sensitivity (TABLE 8). The *Neurospora crassa* bioassay could be omitted from either combination at the expense of missing 1 and 4 tumor-active compounds, respectively. These examples by no means exhaust the possibilities; there are several additional combinations of systems that are equally effective. Of course the frequency of so-called false positives will vary somewhat with different combinations, but it is of interest that the number of false positives does not increase rapidly as additional systems are

TABLE 8

EXAMPLES OF SCREENING COMBINATIONS THAT DETECT THE MAXIMUM NUMBER OF TUMOR-POSITIVE COMPOUNDS*

Screening System†	Number of positive responses					
	Tumor++ compounds		Tumor+ compounds		Tumor− compounds (false positives)	
	Total	Additional‡	Total	Additional‡	Total	Additional‡
11	54		19		51	
9	51	5	14	0	54	13
2	50	3	15	1	36	1
12	42	1	15	1	37	4
Cumulative total§		63		21		69
10	53		15		44	
9	51	3	14	1	54	13
2	50	4	15	1	36	2
12	42	3	15	4	37	8
Cumulative total§		63		21		67

* CCNSC compounds Nos. 20, 44, and 188 were not detected by any of the microbiological or tissue cell culture systems; compounds Nos. 20, 25, 44, 64, and 188 were not detected by any of the microbiological systems.

† See TABLE 1 for identification.

‡ Number of compounds detected by each system *in addition* to those already detected by the preceding system(s) in the combination.

§ All positives detected by combination.

added in combination. For example, with *Tetrahymena pyriformis* (Test System No. 11), 73 of the 89 tumor-active compounds were classified as positive and, at the same time, 51 false positives (TABLE 8) were contributed; yet with the additional 3 systems (*Streptococcus faecalis*, Test System No. 9; *Lactobacillus casei*, Test System No. 2; and *Neurospora crassa*, Test System No. 12; TABLE 1) an additional 11 tumor-active compounds were picked up, and only 18 additional so-called false positives were contributed. The reason for this slow rate of increase obviously is that, with minor variations, all of these 12 microbiological systems, in general, picked up the same false-positive compounds.

Incidence of False Negatives in Combined Microbiological Systems

The tumor-active compounds "missed" in these microbiological systems are:

CCNSC X No. 20	Formamide
CCNSC X No. 25	Colchicine
CCNSC X No. 44	*N*-methylacetamide
CCNSC X No. 64	Strophanthin K
CCNSC X No. 188	*N*-methylformamide

All 5 of these compounds were missed with all of the 16 microbiological assay systems used in these studies. It is of interest to note that formamide (CCNSC X No. 20) has been reported elsewhere to be inhibitory against *Escherichia coli*,[34] again emphasizing the profound influence that differences in the test medium (as well as strain differences in the microorganism used for assay) may have on the results obtained in a bioassay system. An interesting possibility suggested by the observation that these 5 compounds were missed with each of these 16 microbiological systems, representing a variety of diverse metabolic pathways, is that the antitumor activity attributed to these compounds is the result of physiological alteration by the host, rather than a direct cytotoxic effect. Indeed, if this were the case, one would not expect such compounds to be active *in vitro* unless the test microorganism happened to be capable of making the same physiological alteration that results in antitumor activity *in vivo*.

DISCUSSION

The factors affecting evaluation of data such as are presented herein have been discussed by Eagle and Foley[4] in connection with the study of this same series of test compounds in human tissue cell cultures for evidence of cytotoxicity; they will be reviewed here only briefly.

Limitations of Animal Tumor Assay as a Frame of Reference

The results of the present study lend support to the possibility that microbiological assay systems may be of value as a primary screen for the detection of potential antitumor agents. Any screening device has two closely related parameters: *sensitivity*, as evidenced by the number of sought-for compounds picked up, and *reliability*, the number of false positives contributed, that is, those compounds that eventually are shown, upon further study, to be devoid of the desired biological activity. The ultimate criterion for the validation (or repudiation) of a screening procedure designed to detect antitumor agents is, of course, activity against human neoplasms; the inherent premise of any antitumor screening program is that agents that are selectively active against human tumors eventually will be discovered. It is obvious that the ultimate basis for comparison is lacking—and the reasons for this lack are equally obvious. It is necessary, therefore, to evaluate one clearly fallible screening procedure—microbiological assays (or tissue cell cultures)—by comparison with another screening procedure—activity against experimental tumors, which may be equally fallible.

There are several aspects of the animal tumor assay that limit its desirability as a reference for the evaluation of other screening procedures. The usual evaluation of animal tumor assays is neither precise nor objective, and neither false positive nor false negative results can be differentiated, since there is no direct comparison (other than in a few instances) with the ultimate criterion-activity in man. The degree of tumor inhibition accepted as evidence of activity varies with the individual investigator; distinction between specific tumor inhibition and a general cachectic effect often is difficult; dose and route of administration may modify the results obtained; and most of the known tumor-active agents are effective against only a fraction of the tumors tested.[4] Thus, lacking a precise frame of reference, one can hardly expect that the comparisons of sensitivity and reliability that can be made here will prove to have been definitive.

Sensitivity of Microbiological Assays

The obvious limitations on sensitivity inherent in any *in vitro* screen for the detection of antitumor agents, as pointed out by Eagle and Foley,[4] are that (1) of necessity it will fail to detect any compound that must be chemically modified by the host tissues in order to become effective, and that is not similarly modified by the test system; (2) it will fail to detect any agent that acts indirectly, rather than on the test object itself; (3) some compounds may be effective *in vivo* only at such large dosages as to be missed by an *in vitro* procedure based on activity at an arbitrarily fixed and necessarily low concentration; and (4) some intrinsically active compounds may not remain stable in solution for the duration of an *in vitro* test.

Despite these and other factors limiting the efficiency of *in vitro* methods, the sensitivity of a combination of microbiological assay systems is surprisingly good; 95 per cent of the 89 compounds in the series reported to exhibit experimental antitumor activity were detected. Some of the tumor-active agents missed by these microbiological screens (and by tissue cell culture, as well[4]) have exhibited activity in a wide spectrum of animal tumors, and certain of these (formamide and *N*-methylformamide) have appreciable antitumor activity in man. The other missed compounds have been reported as effective only with a fraction of the experimental tumors tested. In the case of these latter compounds, as pointed out by Eagle and Foley,[4] there is no present method short of study in human tumors of determining whether it is the occasional positive result in an experimental tumor or the negative result in other tumors and in microbiological systems that is valid. The converse also is

TABLE 9

EXAMPLES OF TUMOR-NEGATIVE COMPOUNDS HIGHLY ACTIVE *IN VITRO*

CCNSC X No.	Chemical name	Tissue cell cultures*	Microbiological systems†	Experimental tumors†
11	*p*-Anisaldehyde thiosemicarbazone	10^{-5}–10^{-6}	11/12	0/3
35	α-Chloroacetanilide	10^{-5}–10^{-6}	10/12	0/4
55	*n*-Lauryl thiocyanate	10^{-6}–10^{-7}	9/12	0/4
67	Di-*n*-octylamine	10^{-5}–10^{-6}	12/12	0/3
82	Narcotine	10^{-5}–10^{-6}	11/12	0/5
103	Pyronin B	10^{-6}–10^{-7}	12/12	0/6

* ID$_{50}$ in gm./ml.

† Proportion of test systems affected.

true; for example, the compounds mentioned in TABLE 9 were reported to be negative in the few experimental tumor systems studied, but were found to be highly active in microbiological and tissue cell culture tests. Further study may well prove that these compounds are active in other as yet untested experimental tumor systems.

It is important to note that 95 per cent of these tumor-active compounds were detected with as few as four reasonably simple, rapid, and inexpensive microbiological systems. The antitumor data, on the other hand, are a composite of the results obtained in a wide spectrum of experimental tumors, any one of which alone would have detected only a fraction of the tumor-active compounds.

Reliability of Microbiological Assays

This parameter is perhaps less important than sensitivity in a screening procedure designed to detect the maximum number of active agents by the simplest possible methods. Reliability, of course, is intimately related to sensitivity—the more sensitive the screen, the greater the incidence of false positives. Since the criteria that must be used to adjudge positivity are completely arbitrary, sensitivity and reliability can be predetermined to some extent in microbiological screens by the choice of a differential concentration.

The criteria used for positivity in the present analyses were selected to detect the maximum number of tumor-active compounds; consequently, they contributed a rather high proportion of false positives—defined previously, it must be remembered, as those compounds reported not to exhibit activity against the experimental tumors with which they happened to have been tested. Furthermore, the compounds considered, according to available criteria, to be tumor active or tumor negative contain neither false positives nor false negatives, respectively, by *definition* only, since activity in human neoplastic disease is the ultimate criterion. Thus, it cannot be concluded on the basis of any comparison that can be made now that experimental tumor assays pick up fewer false positives or false negatives (in terms of activity in the human disease) than does a microbiological screen. As pointed out in the discussion of the results obtained in tissue cell cultures with these same compounds,[4] the present series was not a random sample, since it included a considerable number of compounds possessing a wide spectrum of biological activities other than antitumor properties. It is not surprising, for example, that substances such as chloramphenicol (CCNSC X No. 89) and benzenesulfonic acid (CCNSC X No. 194) should exhibit antibacterial activity, even though such compounds should be proved to lack antitumor properties. It is evident that the present data may well provide no real information as to the proportion of false positives that would be encountered in a truly random screening of synthetic compounds or crude culture filtrates.

Moreover, one should be prepared to accept a screening procedure that contributes a considerable number of false positives if it also is capable of detecting those agents that eventually prove to be effective in the treatment of human disease. Even assuming a high rate of false positives in a truly random series of compounds, an incidence of 60 to 65 per cent* does not preclude usefulness; if a combination of simple, rapid, and inexpensive microbiological systems can reduce by one third the number of compounds that should be studied further in other experimental systems, it will have served its purpose.

SUMMARY

The data reported herein appear to justify a large-scale exploration of the usefulness of microbiological assay systems for the detection of potential antitumor agents in a routine screening program. It is evident that the majority (95 per cent) of those compounds adjudged to be tumor-active in animal tumor assays can be detected by virtue of their inhibitory effects on microorganisms, using as few as 4 selected bioassay systems. It is equally evident that there are a considerable number of false-positive (60 to 65 per cent) and some false-negative (5 per cent) results, as compared with the available experimental antitumor data, among the compounds herein studied. Since a similar situation may prove to exist when effectiveness in animal tumor assays is compared with effectiveness in human neoplasia, such results do not detract from the potential usefulness of microbiological screens.

The sixteen microbiological assay systems considered here present a considerable number of highly effective combinations, but by no means limit the possible choice—there being many other systems that may be equally effective, or perhaps more so.

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* This figure is computed on the tumor-negative compounds in this series. If computed on the total series of 180 compounds tested, the rate of false positives would be only 30 to 35 per cent.

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Discussion of the Paper

Philip S. Thayer (*Arthur D. Little, Inc., Cambridge, Mass.*): In a study such as this, secondary points often contribute to the interest and significance of the results. I propose to comment on two features of the *Neurospora* work that illustrate this point.

In our work with *Neurospora*, we did not make a concerted effort to solubilize all compounds tested. As a result, 106 of the 198 compounds tested were rated as soluble, that is, they dissolved readily at 10^{-3} (or 1 mg./ml.), whereas 44 were only partially soluble at this concentration, and the remaining 48 were essentially insoluble.

It is pertinent therefore to analyze the results of the inhibition tests with

TABLE 1

NEUROSPORA ASSAY: DISTRIBUTION OF COMPOUNDS BY SOLUBILITY AND ACTIVITY CLASSES

Tumor class	Solubility	No.	Active at:*					
			>3	3–4	4–5	5–6	6–7	7–8
++, +	+	45	19	9	6	7	3	1
++, +	−	44	13	13	5	9	3	1
−	+	53	33	17	0	3	0	0
−	−	37	20	5	10	1	1	0

* Concentrations are expressed as negative logarithms of ID_{50} range; for example, 3–4 = 10^{-4}–10^{-3} gm./ml.

reference to (1) the solubility classes, (2) the *Neurospora* activity classes, and (3) the tumor activity classes, as presented by Foley. TABLE 1 shows this analysis in terms of the number of compounds falling into each class. The tumor-positive compounds were divided nearly equally between our soluble and insoluble classes (45 and 44); surprisingly, a slightly greater percentage of the insolubles was detected by *Neurospora* as true positives (31 of 44, or 70 per cent, versus 26 of 45, or 58 per cent). Furthermore, when the distribution of the active compounds among the ID_{50} classes is considered, the insoluble compounds show a very similar distribution. It may be concluded that, at least in the present limited series of compounds, the results would not be altered significantly if all compounds had been initially solubilized, since their activity against *Neurospora* is sufficiently high to show after dilution to the level of solubility.

A similar inspection of the results with the tumor-negative compounds (the last two lines of the table) shows that the percentage of active compounds (false positives) among the insoluble compounds is slightly higher than in the soluble compounds (46 per cent compared to 38 per cent). The distribution of active compounds shows in *both* groups the same effect that Foley commented on, that is, the tumor-negative compounds are, on the average, less active than the tumor positives.

To turn to a second supplementary topic, let us next consider the 7 styrylquinoline compounds (X-16, 36, 76, 87, 102, 135, and 196) tested in this study. *Neurospora* was inhibited by all of them at concentrations of 100 μg./ml. or less, and 6 of the 7 at 10 μg./ml. or less. None of the other microbiological systems was affected so consistently. For that reason, it was decided to test the effects of other styrylquinoline compounds, of which a large number have been synthesized by Carl Tabb Bahner and his associates at Carson-Newman College (TABLE 2).* I should like to point out a few points of the structure-activity relationships for *Neurospora*.

* This work was done under grants to Bahner from The Damon Runyon Memorial Fund for Cancer Research Inc., The Research Corporation, and the American Cancer Society, Inc., all in New York, N. Y., and The Medical Research Foundation, Philadelphia, Pa. Syntheses were performed by Bahner's associates, including George Browder, Clarence Cook, John Dale, John Ellis, John Fain, Edgar Franklin, Wilfred Lyke, Albert Myers, Ronald Nations, Robert Neely, Edwin Pace, Robert Prevost, Mary West, and Joan Wilson.

TABLE 2

TOXICITY OF STYRYLQUINOLINES TO *NEUROSPORA CRASSA*, WILD TYPE 4A*

Compound	No. tests	ED$_{50}$ class (γ/ml.)
4-(*p*-Dimethylaminostyryl)-3-methylquinoline	2	~100
4-(*p*-Dimethylaminostyryl)-6-methylquinoline	2	P100
4-(*p*-Dimethylaminostyryl)-7-methylquinoline	2	P100
4-(*p*-Dimethylaminostyryl)-8-methylquinoline	2	~100
4-(*p*-Dimethylaminostyryl)-3,6-dimethylquinoline	3	~10
4-(*p*-Dimethylaminostyryl)-3,8-dimethylquinoline	2	~100
4-(*p*-Dimethylaminostyryl)-5,7-dimethylquinoline	2	P100
4-(*p*-Dimethylaminostyryl)-5,8-dimethylquinoline	2	P100
4-(*p*-Dimethylaminostyryl)-6,7-dimethylquinoline	2	P100
4-(*p*-Dimethylaminostyryl)-6,8-dimethylquinoline	2	P100
4-(*p*-Dimethylaminostyryl)-7,8-dimethylquinoline	2	P100
4-(*p*-Dimethylaminostyryl)-6-bromoquinoline	2	>100
4-(*p*-Dimethylaminostyryl)-6-chloroquinoline	1	10–100
4-(*p*-Dimethylaminostyryl)-7(or 5)-chloroquinoline	1	10–100
4-(*p*-Dimethylaminostyryl)-8-chloroquinoline	1	P100
4-(*p*-Dimethylaminostyryl)-6-iodoquinoline	1	>100
4-(*p*-Dimethylaminostyryl)-2,3-benzoquinoline	1	P100
4-(*p*-Dimethylaminostyryl)-5,6-benzoquinoline	1	10–100
4-(*p*-Dimethylaminostyryl)-7,8-benzoquinoline	1	P100
4-(*p*-Dimethylaminostyryl)-8-phenylquinoline	1	~100
4-(*p*-Dimethylaminostyryl)-3-methyl-6-iodoquinoline	1	10–100
4-(*p*-Dimethylaminostyryl)butadienylquinoline	1	P100
4-(*p*-Dimethylaminoanil)quinoline	1	10–100
4-(*p*-Dimethylaminostyryl)-5,6-benzoquinoline methiodide hydrate	2	1–2.5
4-(*p*-Dimethylaminostyryl)quinoline methiodide	2	5–10
4-(*p*-Dimethylaminostyryl)-3-methylquinoline methiodide	2	5–10
4-(*p*-Dimethylaminostyryl)butadienylquinoline methiodide	2	0.5–1
4-(*p*-Dimethylaminostyryl)quinoline dimethiodide	1	10–100
4-(*p*-Dimethylaminostyryl)quinoline ethiodide	2	5–10
4-(*p*-Nitrostyryl)quinoline methiodide	1	~100
4-Aminostyrylquinoline	3	5–10
4-(Ethylbenzylaminostyryl)quinoline†	4	prob. 0.5–1
4-(*p*-Diethylaminostyryl)quinoline ethiodide	2	2.5–5
4-(*p*-Dimethylaminoanil)pyridine	1	10–100
4-(*p*-Dimethylaminostyryl)pyridine	1	>100
4-(*p*-Dimethylaminostyryl)pyridine ethiodide	1	10–100
4-(*p*-Dimethylaminostyryl)pyridine methiodide	1	10–100
2-(*p*-Dimethylaminostyryl)pyridine methiodide	1	10–100
2-(*p*-Dimethylaminoanil)pyridine ethiodide	1	~100
2-(*p*-Dimethylaminostyryl)quinoline	2	>100
2-(*p*-Dimethylaminostyryl)benzothiazole	1	>100
2-(*p*-Dimethylaminostyryl)-5,6-benzoquinoline	1	P100
2-Styrylquinoline methiodide	1	P100
2-(*p*-Dimethylaminostyryl)quinoline methiodide	3	10–50
2-(*p* Dimethylaminostyryl)-6-methylquinoline methiodide	2	2.5–5
2-(*p*-Dimethylaminostyryl)benzothiazole methiodide	3	2.5–5
2-(*p*-Dimethylaminostyryl)-5,6-benzoquinoline methiodide hydrate	2	~2.5
2-(*p*-Methoxystyryl)quinoline methiodide	1	10–10
2-(*p*-Methoxystyryl)quinoline dimethiodide	1	10–100
2-(3,4-Dimethoxystyryl)quinoline methiodide	1	>100
2 (3,4-Methylenedioxystyryl)quinoline methiodide	1	P100
2 (3,4-Methylenedioxystyryl)pyridine methiodide	1	>100
2 Styrylpyridine methiodide	1	>100
2-Methoxystyrylpyridine methiodide	1	>100
2-(*p*-Dimethylaminostyryl)quinoline ethiodide	2	~2.5
2-(*p*-Diethylaminostyryl)quinoline ethiodide	3	2.5–5
2-(*p*-Diethylaminostyryl)quinoline methiodide	3	10–50
2-(*p*-Diethylaminostyryl)pyridine methiodide	1	10–100

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* Assay measures concentration range within which inhibitor produces 50 per cent inhibition of growth. Growth: 48 hours, at 32° C. All test materials were dissolved or suspended in 0.1 N HCl and diluted with sterile water or buffer for addition. P100 = Partial inhibition at 100 γ /ml.

† Very erratic results.

TABLE 2—*Continued*

Compound	No. tests	ED_{50} class (γ/ml.)
2,6-Ethoxystyryl pyridine methiodide	1	>100
2-Methyl-6-(2-α-thienylvinyl)pyridine methiodide	1	10–100
2-(*p*-Acetylaminostyryl)pyridine methiodide	1	10–100
2-(*p*-Dimethylaminostyryl)-5-methylpyrazine methiodide	1	10–100
2-(3,4-Diethoxystyryl)quinoline methiodide	1	10–100
2-(3,4-Dichlorostyryl)quinoline methiodide	1	10–100
3-(*p*-Dimethylaminostyryl)isoquinoline methiodide	1	10–100
1-(*p*-Dimethylaminoanil)naphthalene	1	10–100
1-(*p*-Dimethylaminostyryl)naphthalene	1	>100
1-(*p*-Diethylaminostyryl)isoquinoline	1	~100
4-Methylquinoline methiodide	1	>100
4-(*p*-Dimethylaminostyryl)quinazoline	2	0.5–1
4-(*p*-Diethylaminostyryl)quinoline dihydrochloride	1	0.25–0.5
4-(*p*-Diethylaminostyryl)quinoline	3	0.05–0.1
4-(*p*-Dimethylaminostyryl)quinoline	3	0.5–1.0
4-(*p*-Dimethylaminostyryl)quinoline methachloride	1	1–2.5
2-(*p*-Diethylaminostyryl)quinoline	3	~100
2-(*p*-Diethylaminostyryl)-6-methylquinoline	2	25–50
2-*m*-Nitrostyrylquinoline	1	>100

The most active compound was the *p*-diethylaminostyryl compound, which gave 50 per cent inhibition at concentrations between 0.05 and 0.1 μg./ml. The corresponding dimethylamino compound was only slightly less active. Other changes that permitted retention of activity below 1 μg./ml. include the substitution of a quinazoline ring, of butadienylquinoline, and of a benzyl group for one of the ethyls. Activity was reduced only tenfold by some other changes, including the formation of the methiodide salt of the parent compound, the substitution of the 5,6 benzoquinoline ring, the presence of a styrylamino group without alkyl substituents, only one of the various methylations performed in the quinoline ring—at the 3 position and, finally, the shift of the styryl group to the 2 instead of the 4 position on the quinoline ring. Any other changes resulted in much greater decreases in activity. Although Bahner's data have not been published, he has informed me that the results with *Neurospora* are for the most part consistent with the tumor data.

ESMOND E. SNELL (*University of California, Berkeley, Calif.*): I should like to mention the surprising correlation between the results obtained with *Tetrahymena* and the yeast, *Saccharomyces carlsbergensis*. In the past, considerable emphasis has been placed on the utility of protozoa in that they belong to the animal kingdom. Perhaps this should be clarified, in view of the fact that *S. carlsbergensis* picked up just as many of these toxic compounds as did the protozoan organism.

UTILITY OF DRUG-RESISTANT ORGANISMS IN CANCER CHEMOTHERAPY STUDIES

By F. M. Schabel, Jr.

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For several years a number of laboratories have been using microorganisms in searches for new potentially useful anticancer agents and in studies on the possible modes of action of demonstrated anticancer agents. The published results of these studies attest to the usefulness of microorganisms and microbiological techniques in cancer chemotherapy research. A number of examples can be cited in which anticancer compounds were first suggested for testing for anticancer activity on the basis of inhibition of microorganisms.[1]

For some time my associates and I have been using microorganisms in a variety of ways in our cancer chemotherapy research program at Southern Research Institute. While we recognize that it has not been possible in all cases to show a positive correlation between the action of drugs on the metabolism of microorganisms and on the metabolism of normal and neoplastic mammalian cells, nevertheless we feel that the use of microorganisms in cancer chemotherapy studies has been and will continue to be very useful.

Microorganisms, like neoplastic mammalian cells, sometimes develop resistance to chemotherapeutic agents, particularly during continued exposure to the agent; I propose to discuss the usefulness of some anticancer drug-resistant microorganisms in several active areas of cancer chemotherapy research.

We have a primary screening laboratory operating under contract with the Cancer Chemotherapy National Service Center. About 80 per cent of our total primary screening activity is involved with crude antibiotic beers and about 20 per cent with synthetic organic compounds. Among these materials, a small percentage shows reproducible anticancer activity against one or more of the mouse tumors used. Provided the active material is a crude beer, the first observation of anticancer activity gives us little if any indication of the probable type of agent the beer may contain or whether or not the active principle is similar to or different from presently known naturally occurring anticancer agents such as azaserine, netropsin, actinomycin, fumagillin, sarkomycin, or others. Further, we do not know whether the active principle may be a purine antagonist, an antifolic, or otherwise similar, as regards its metabolite antagonism, to any presently known agent. We believe that, with few possible exceptions, new anticancer agents differing in their biochemical modes of action from agents presently known would be of greater interest than those with similar or identical modes of action to known agents.

Use of Drug-Resistant Microorganisms to Separate New from Known Naturally Occurring Anticancer Agents

We are attempting to distinguish new agents with modes of action differing from known agents by using the drug-resistant and drug-sensitive strains of

TABLE 1

DRUG-RESISTANT AND DRUG-SENSITIVE MICROORGANISMS USED IN SECONDARY SCREENING

Microorganism	Degree of resistance compared to drug-sensitive
E. coli/parent drug-sensitive	
E. coli/sulfanilamide-resistant	100×
E. coli/azaserine-resistant	>10,000×
E. coli/netropsin-resistant	75×
S. faecalis/parent drug-sensitive	
S. faecalis/A-Methopterin-resistant*	>100,000×
S. faecalis/6-mercaptopurine-resistant*	50×
S. faecalis/8-azaguanine-resistant*	100×
S. faecalis/actinomycin D-resistant	10×

* Obtained from Dorris Hutchison.

Escherichia coli and *Streptococcus faecalis* shown in TABLE 1. The drug-resistant strains were all selected from subcultures carried through multiple transfers in liquid media containing increasing concentrations of the inhibitor.

Briefly, the procedure used is as follows. The organisms are incorporated into chemically defined solid media,* and the beer or synthetic, previously shown to have anticancer activity, is placed on the surface of the seeded agar on filter paper disks moistened with known concentrations of the synthetics or known dilutions of the beers. Following suitable incubation, the cultures are examined for inhibition of growth by the new agents. Inhibition of the parent drug-sensitive line of an organism and failure to inhibit a drug-resistant line of the same organism suggest that the new agent is identical with or similar in mode of action to that agent to which the organism is resistant.

In TABLE 2 are shown some results obtained with known concentrations of azaserine.

I understand that 20 μg./ml. of azaserine is common in crude azaserine beers. If so, the azaserine-resistant and azaserine-sensitive strains of *E. coli* will make preliminary identification of azaserine-containing crude beers an easy matter; in fact, using this procedure, these organisms could identify azaserine in an active beer containing less than 1 μg./ml. of azaserine.

TABLE 3 shows results of a similar test with 6-diazo-5-oxo-L-norleucine (DON). Here it can be seen that the azaserine-sensitive and azaserine-resistant *E. coli*, while not separating azaserine and DON, permit the deduction that these agents have, at least in this microbial system, similar biochemical modes of action.

Similar results are seen with the other drug-resistant organisms listed in TABLE 1, when each is similarly exposed to the drug to which it is resistant.

It will be noted that our collection of bacteria resistant to naturally occurring anticancer agents is quite limited. Our actinomycin D-resistant *S. faecalis* has very limited resistance, but certainly is metabolically different from the actinomycin D-sensitive *S. faecalis*, as will be shown. We do not have an

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* *E. coli* medium—basal medium of Davis and Mingioli;[2] *S. faecalis* medium—medium of Flynn *et al.*, without purines or pyrimidines.[3]

TABLE 2

INHIBITION OF AZASERINE-SENSITIVE AND AZASERINE-RESISTANT *E. COLI* BY AZASERINE

Total azaserine on test disk*	Radius of zone of inhibition (cm.) *E. coli*	
	Azaserine-sensitive	Azaserine-resistant
μg.		
(Diluted in negative beer)		
200.0	3.0	0
10.0	2.3	0
1.0	1.6	0
0.1	1.4	0
0.01	0.6	0
(Diluted in saline)		
0.1	1.4	0
0.05	1.1	0
0.01	0.7	0

* Whatman No. 5, 1 cm. in diameter. Each disk contains about 0.01 ml. of fluid.

TABLE 3

INHIBITION OF AZASERINE-SENSITIVE AND AZASERINE-RESISTANT *E. COLI* BY 6-DIAZO-5-OXO-L-NORLEUCINE (DON)

Total DON on test disk*	Radius of zone of inhibition (cm.) *E. coli*	
	Azaserine-sensitive	Azaserine-resistant
μg.		
(Diluted in negative beer)		
200.0	3.0	0
10.0	2.5	0
1.0	2.0	0
0.5	1.5	0
0.1	0	0
(Diluted in saline)		
200.0	3.0	0
10.0	2.2	0
1.0	1.8	0
0.5	1.2	0
0.1	0	0

* Whatman No. 5, 1 cm. in diameter. Each disk contains about 0.01 ml. of fluid.

organism with acquired resistance to fumagillin, sarkomycin, carzinophilin, or any of the other naturally occurring anticancer agents reported recently. These are certainly needed for maximum effectiveness of this procedure for separating new from known anticancer agents.

Use of Drug-Resistant Microorganisms in a Search for New Agents with Selective Toxicity for Drug-Resistant Neoplastic Cells

The development of resistance to initially effective chemicals by animal and human neoplastic cells, particularly leukemic cells, is probably one of the

TABLE 4
INHIBITORY ACTIVITY OF SOME ANTITUMOR ANTIBIOTIC BEERS FOR DRUG-SENSITIVE AND DRUG-RESISTANT *S. FAECALIS*

Beer No.	Antitumor activity	Radius of zone of inhibition (cm.) *S. faecalis*				
		Parent drug-sensitive	A-Methopterin-resistant	6-Mercaptopurine-resistant	8-Azaguanine-resistant	Actinomycin D-resistant
A-33	Sa 180, Ca 755	1.5	0	1.5	2.0	1.5
A-34	Ca 755	1.0	0.6	0.6	1.0	0.5
A-90	Sa 180, Ca 755	1.5	1.5	1.5	1.5	1.5
A-77	Sa 180	1.0	0	1.0	1.0	0
A-72	Sa 180, Ca 755	0	0	0	0.8	0
A-86	Sa 180	1.5	1.5	1.8	1.5	1.5
A-54	Sa 180	1.7	1.8	2.0	1.8	2.0
A-89	Sa 180	0	0.7	0.7	0.7	0.5
A-38	Sa 180	1.0	0.7	1.2	1.3	1.0

most important reasons why the presently known anticancer agents are palliative but not curative.

The demonstration of drugs with selective toxicity for drug-resistant neoplastic cells would suggest that they might be useful in treating tumors that had become resistant to initially effective drugs or in combination with drugs presently known to have temporary anticancer activity.

If we assume that metabolic similarity does exist between microorganisms and neoplastic cells resistant to the same drug, then drug-resistant bacteria can be very useful in searches for agents with selective toxicity for drug-resistant neoplastic cells. Currently we are screening new synthetic and natural materials that show reproducible carcinostatic activity for selective toxicity for drug-resistant microorganisms.

Some examples of bacterial inhibitions seen with active antitumor beers against several strains of *S. faecalis* are shown in TABLE 4. In this test, filter-paper disks moistened with about 0.01 ml. of the crude beer were placed on the surface of solid media seeded with each organism, and the inhibition zones were noted after suitable incubation.

A number of interesting and potentially useful results are shown. It will be noted that A-33 and A-77 both fail to inhibit the A-Methopterin-resistant organism while inhibiting the parent drug-sensitive organism. I have stated previously that the cross resistance to a new agent by an organism resistant to a known anticancer agent would make the new material of less interest than one presumed to be different in mode of action from known anticancer agents. Generally, I believe this to be true. However, the possibility that these beers may contain naturally occurring materials possessing antifoliclike activity makes them of particular interest.

Both the A-Methopterin-resistant and the actinomycin D-resistant organisms are cross resistant to A-77. It is unlikely that the active principle in this beer is actinomycin D, because in our experience the A-Methopterin-resistant organism is quite sensitive to actinomycin D. Perhaps the cross resistance of both of these organisms to an active principle in this beer can be explained, and some speculations might cause us to be suspicious concerning the mode of action of the active principle. Foley[4] has reported that actinomycin D inhibition of *Lactobacillus fermenti* can be reversed by thymine, orotic acid, adenine, and methionine, in addition to pantothenate, pyruvate, and oxalacetate. A-Methopterin inhibition of *S. faecalis* can be prevented by the addition of purines and thymine to the growth medium.[5] Apparently both A-Methopterin and actinomycin D inhibition, of bacteria at least, interfere with the biosynthesis of some common essential metabolites and, if this is true, then the observation of the cross resistance to A-77 of the strains of *S. faecalis* resistant to both A-Methopterin and actinomycin D could be expected.

A-72 is of considerable interest because it is selectively toxic only for the 8-azaguanine-resistant strain of *S. faecalis*. On the basis of this observation, we think this agent should be studied for activity against 8-azaguanine-resistant neoplasms and in combination with 8-azaguanine against purine antagonist-sensitive tumors.

At the concentrations present in the crude beer, A-89 is not inhibitory for the parent drug-sensitive strain of *S. faecalis*, but it does inhibit the drug-resistant organisms. Present knowledge indicates that drug-resistant cells, microbial or mammalian, may in some instances be characterized by deficiencies in their metabolic synthesizing abilities; therefore, increased sensitivity of these organisms to metabolitic inhibitors might be expected. Consequently, agents such as A-89 should be tested for selective toxicity to drug-resistant neoplasms and also in combination with presently known effective anticancer agents.

TABLE 5 shows the activity of these same active beers against the several strains of *E. coli* we are using. It is of interest to note that only one of these active beers was inhibitory for any of the strains of *E. coli*. This is not surprising, of course, since *E. coli* is known to be a much more versatile organism than *S. faecalis*, being able to synthesize all of its cellular constituents from very simple substrates. The sensitivity of the azaserine-resistant strain of *E. coli* to A-38, in addition to suggesting its possible usefulness in treating

TABLE 5
INHIBITORY ACTIVITY OF SOME ANTITUMOR ANTIBIOTIC BEERS FOR DRUG-SENSITIVE AND DRUG-RESISTANT *E. COLI*

Beer No.	Antitumor activity	Radius of zone of inhibition (cm.) *E. coli*			
		Parent drug-sensitive	Azaserine-resistant	Netropsin-resistant	Sulfanilamide-resistant
A-33	Sa 180, Ca 755	0	0	0	0
A-34	Ca 755	0	0	0	0
A-90	Sa 180, Ca 755	0	0	0	0
A-77	Sa 180	0	0	0	0
A-72	Sa 180, Ca 755	0	0	0	0
A-86	Sa 180	0	0	0	0
A-54	Sa 180	0	0	0	0
A-89	Sa 180	0	0	0	0
A-38	Sa 180	0	1.5	0	0

azaserine-resistant tumors or treating azaserine-sensitive tumors in combination with azaserine, suggests that it might be useful as an assay tool in following fractionation of this beer and isolation of the active principle. Application of such an organism to this purpose presupposes that the antitumor and antibacterial agent are the same; for the moment, this point is speculative. However, this example and the several shown in TABLE 3 suggest the possibility that drug-resistant microorganisms might be useful for this purpose.

Similar selective toxicity of crystalline compounds for drug-resistant microorganisms can be demonstrated. Some years ago we reported that sulfanilamide-resistant *E. coli* were more sensitive to inhibition by purine antagonists (6-mercaptopurine, 6-chloropurine, 8-azaguanine)[6] than the parent sulfanilamide-susceptible *E. coli*. We have seen recently that the A-Methopterin-resistant *S. faecalis* is strikingly more sensitive to 6-mercaptopurine than is the A-Methopterin-sensitive strain of *S. faecalis*.

These observations do not correlate with Leukemia 1210 (L 1210). For example, A-Methopterin-resistant L 1210 is not selectively inhibited by 8-azaguanine or 6-mercaptopurine. However, the purine-antagonist resistant (8-azaguanine and 6-mercaptopurine) lines of L 1210 are significantly more sensitive to A-Methopterin;[7] also, acute leukemia in adults which has become refractory to 6-MP during 6-MP therapy shows a higher incidence of favorable response to A-Methopterin than similar cases treated with A-Methopterin without prior 6-MP treatment.[8, 9] While the direct correlation between the sensitivity of A-Methopterin-resistant and sulfanilamide-resistant bacteria and neoplastic cells to purine antagonists is lacking, the over-all relationship between these two types of antagonists suggests the testing of folic acid antagonist-resistant cells with purine antagonists, and vice versa. Perhaps, if our foresight were better, these new observations on selective toxicity of new agents for drug-resistant microorganisms would suggest new effective combinations of anticancer drugs.

TABLE 6 illustrates the recently observed selective toxicity of a crystalline antileukemic agent for drug-resistant microorganisms.

TABLE 6

SELECTIVE TOXICITY OF A CRYSTALLINE ANTIBIOTIC ANTILEUKEMIC AGENT FOR DRUG-RESISTANT *S. FAECALIS*

Total compound on disk	Radius of zone of inhibition (cm.) *S. faecalis*				
	Parent drug-sensitive	A-Methopterin-resistant	6-Mercaptopurine-resistant	8-Azaguanine-resistant	Actinomycin D-resistant
µg.					
3.0	1.3	2.0	2.5	2.5	1.8
1.5	1.2	1.8	2.5	2.5	1.3
1.0	1.2	1.0	2.5	1.9	1.2
0.8	1.0	1.0	2.5	1.7	1.0
0.6	0.8	0.8	2.5	1.3	0.7
0.3	0.7	0.8	1.6	1.3	0.6
0.1	0	0	1.0	1.0	0

The purine antagonist-resistant strains of *S. faecalis* are consistently more sensitive to inhibition with this compound than the drug-sensitive strain, the A-Methopterin-resistant strain, or the actinomycin D-resistant strain. It does not inhibit the several strains of *E. coli* at the same drug levels. If the observations recorded in TABLE 6 can be correlated with *in vivo* activity, we can deduce that this compound is probably not an antifolic or a purine antagonist. Neither is it an actinomycin. Since it does not inhibit *E. coli* or any of the drug-resistant strains, it is neither azaserinelike nor netropsinlike. Preliminary metabolite reversal studies with drug-sensitive *S. faecalis* indicate that its inhibitory action can be reversed by a mixture of vitamins. Thus it can be seen that a number of interesting and potentially useful observations regarding the biological activity of this compound have been quickly and easily obtained using drug-resistant microorganisms, the only reservation concerning the relationship of these observations to *in vivo* activity being the as yet undemonstrated correlation between *in vitro* activity against microorganisms and *in vivo* activity in tumor-bearing animals. However, since this is a crystalline material, direct correlation between the *in vitro* and *in vivo* observations is probable.

It appears probable that the Cancer Chemotherapy National Service Center's present, greatly accelerated primary screening program will soon reveal more interesting and potentially useful leads than can be evaluated carefully, if indeed it has not already done so. We think that the use of drug-resistant microorganisms in secondary screening of the leads revealed in the primary screen offers one way to obtain some data that may be of real help to those whose responsibility it will be to decide which of the many will receive intensive study.

Use of Drug-Resistant Microorganisms in Studies on the Mode of Action of Anticancer Agents

In addition to the uses of drug-resistant microorganisms in secondary screening of new anticancer agents, we have been interested in studying the biochemical mode of action of known anticancer agents, using drug-resistant microorganisms. Brockman in our laboratories and Hutchison and Balis of the Sloan-Kettering Institute have been working on mechanisms of drug resistance to anticancer agents, using drug-resistant microorganisms and drug-resistant mouse leukemias. Brockman has provided me with some of the data he has collected, and I shall summarize them briefly to illustrate the usefulness of drug-resistant microorganisms in mechanism studies with anticancer agents.

Results published to date[10] indicate that *S. faecalis* and Leukemia 1210 convert 8-azaguanine to 8-azaguanylic acid, which may be the active form of the antagonist. The 8-azaguanine-resistant *S. faecalis* and Leukemia 1210, on the other hand, do not convert this fraudulent purine to the ribotide. Failing to do so, both the 8-azaguanine-resistant bacteria and the 8-azaguanine-resistant leukemic cells are able to maintain growth in the presence of concentrations of 8-azaguanine that will severely inhibit the drug-sensitive organisms or leukemic cells. FIGURE 1 shows the labeled metabolic end products

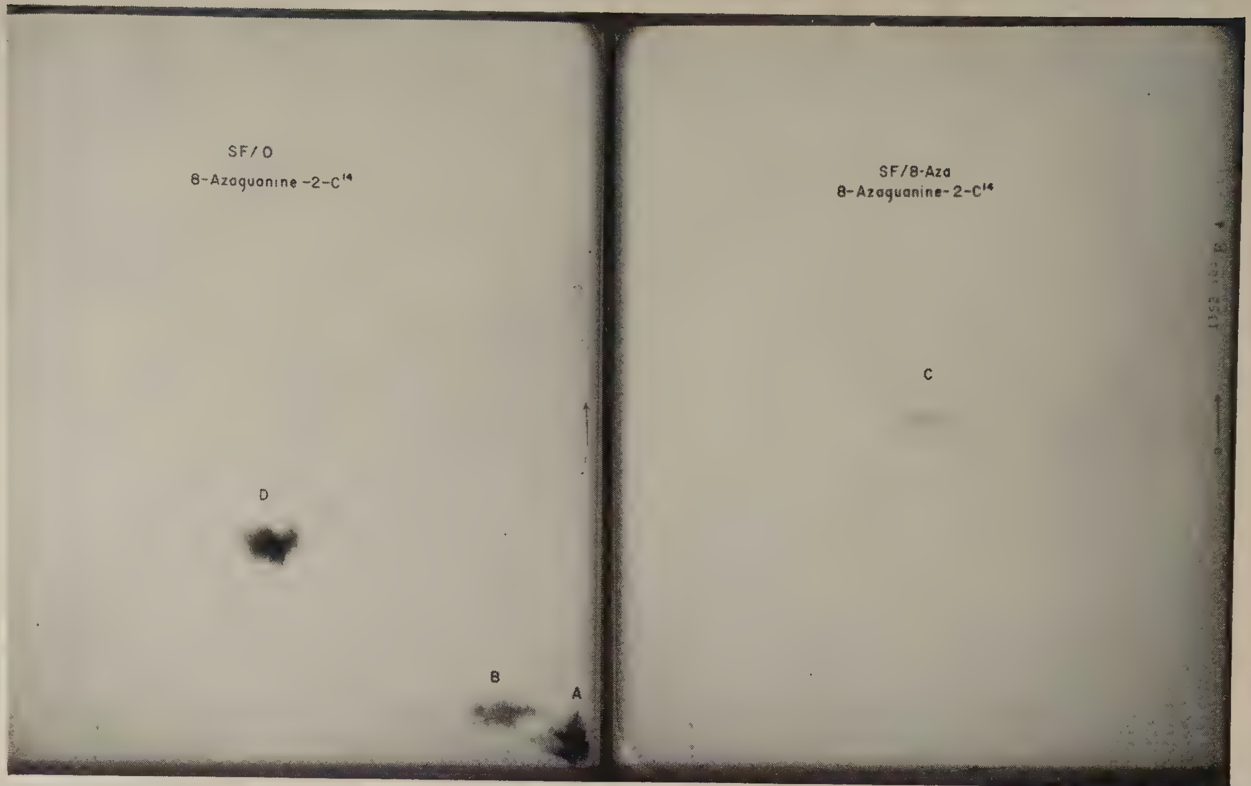

FIGURE 1. Two-dimensional paper chromatogram-autoradiograms of extracts of SF/0 and SF/8-Aza exposed to 8-azaguanine-2-C^{14}. The radioactive spots have been identified by chromatographic and electrophoretic methods as follows: (D) 8-azaguanosine, (B) 8-azaguanylic acid, (A) 8-azaguanosine diphosphate and/or triphosphate, and (C) 8-azaguanine.

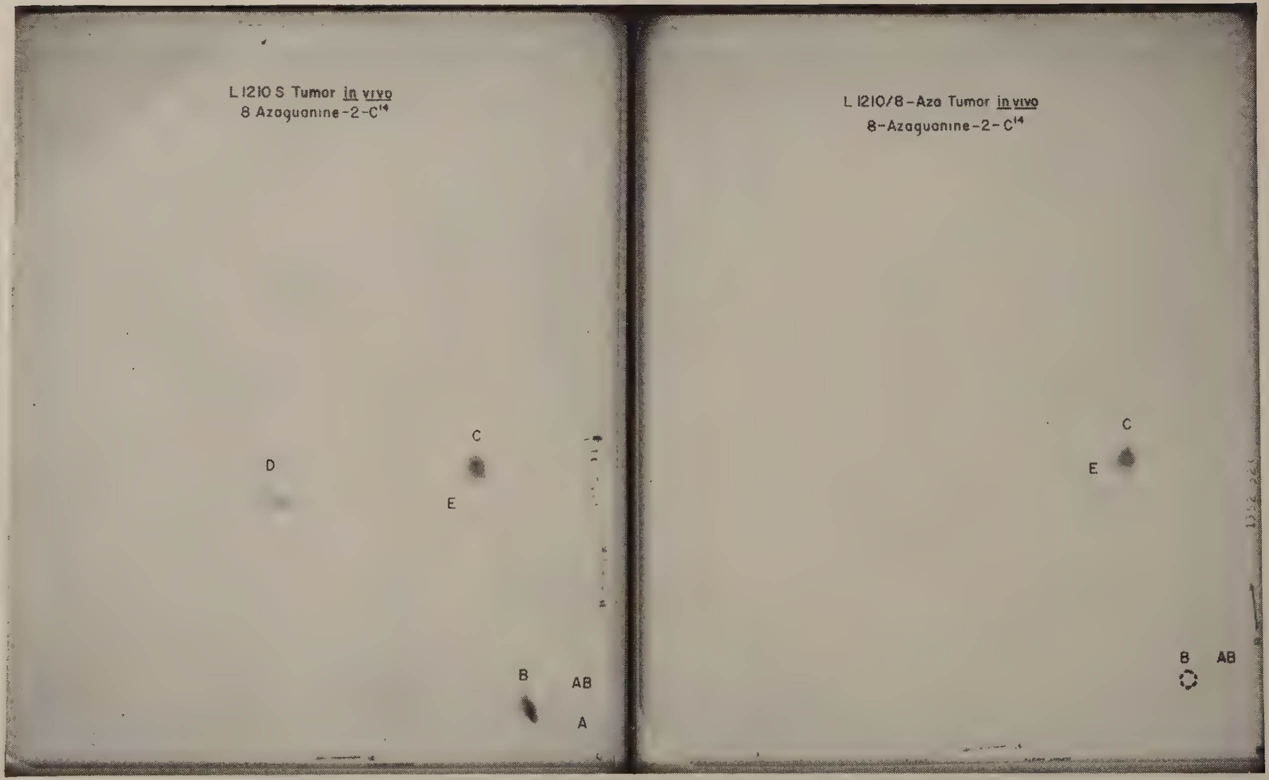

FIGURE 2. Two-dimensional paper chromatogram-autoradiograms of extracts of L 1210S and L 1210/8-Aza taken from the same host mouse treated with 8-azaguanine-2-C^{14}. The identity of the radioactive spots is the same as in FIGURE 1, except for spot E, which is as yet unidentified.

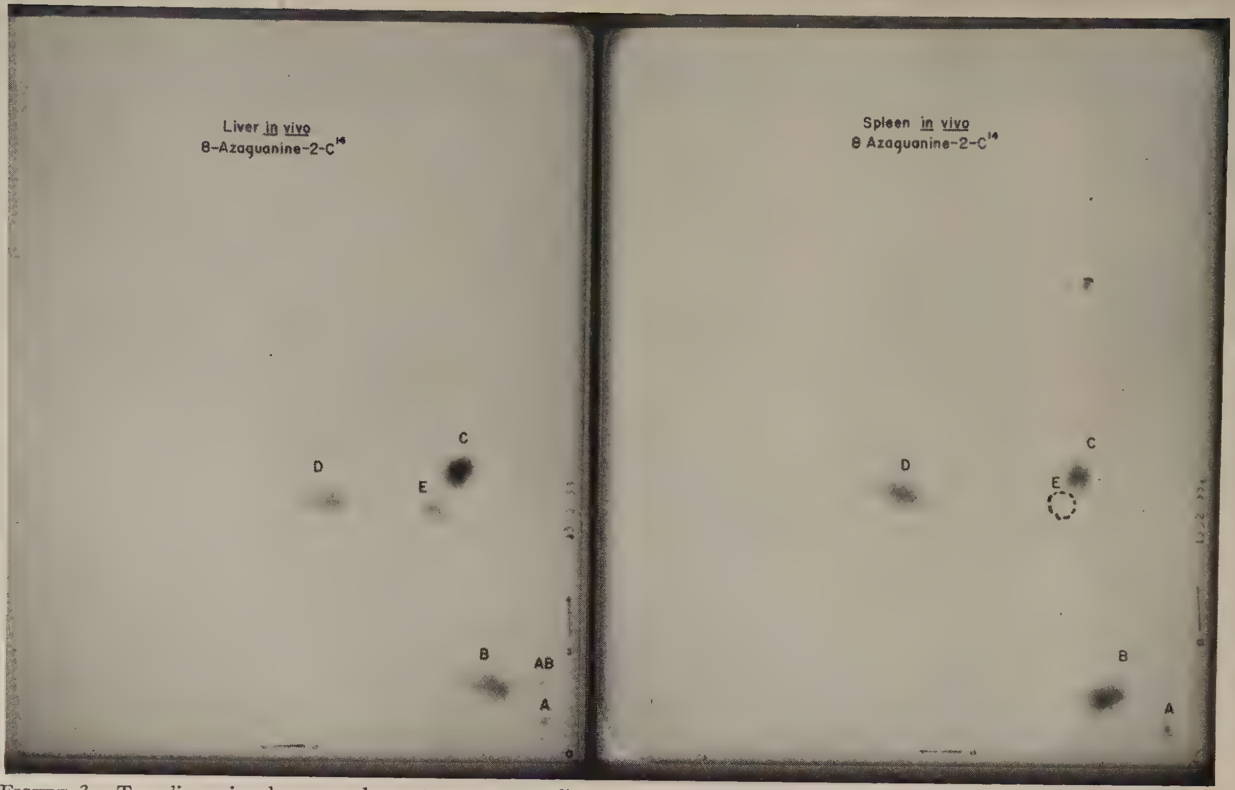

FIGURE 3. Two-dimensional paper chromatogram-autoradiograms of extracts of spleen and liver taken from the same mouse treated with 8-azaguanine-2-C^{14}. The identity of the radioactive spots is the same as in FIGURES 1 and 2.

TABLE 7

SUMMARY OF METABOLISM OF NATURAL AND FRAUDULENT PURINES BY SUSCEPTIBLE OR RESISTANT CELLS

SF/0	Reaction	SF/8-Aza	SF/MP
+	Hx → HxRP	0	0
+	6-MP → 6-MPRP	0	0
+	Gu → GuRP	0	0
+	8-AzaGu → 8-AzaGuRP	0	0
L 1210*	Reaction	L 1210/8-Aza*	L 1210/MP
+	8-AzaGu → 8-AzaGuRP	0	0

* Differences in metabolism of 8-azaguanine by L 1210S and L 1210/8-Aza observed in subcutaneous tumors *in vivo*, in ascites cells *in vivo*, and in ascites cells *in vitro*.

obtained when actively growing drug-sensitive *S. faecalis* (SF/0) and 8-azaguanine-resistant *S. faecalis* (SF/8-Aza) are incubated with 8-azaguanine-2-C^{14}. Hot aqueous-alcohol extracts of these cells were prepared, and two-dimensional paper chromatogram-autoradiograms were made. From these autoradiograms, it is obvious that the 8-azaguanine-sensitive organisms extensively metabolize 8-azaguanine, whereas the drug-resistant organisms fail to do so.

FIGURE 2 shows results of a similar comparison of 8-azaguanine-sensitive and 8-azaguanine-resistant Leukemia 1210 growing bilaterally in the same 8-azaguanine-treated host mouse. FIGURE 3 shows similarly prepared autoradiograms of liver and spleen from the host mouse from which the tumors in FIGURE 2 were taken. It is apparent that normal tissue metabolism of 8-azaguanine is identical to that of the 8-azaguanine-sensitive Leukemia 1210.

TABLE 7 summarizes Brockman's results to date using drug-resistant and drug-sensitive *S. faecalis* and Leukemia 1210. These results indicate that the drug-resistant cells fail to metabolize the natural purines, hypoxanthine and guanine, to the corresponding ribotides; similarly, they fail to metabolize the fraudulent purines, 8-azaguanine and 6-mercaptopurine, to the corresponding ribotides, while the drug-sensitive cells are able to carry out these metabolic conversions.

It is of interest to know that the Russians are screening antibiotics for anticancer activity with mutant bacteria. The organisms under study are characterized by deficient respiration and possibly other alterations in cell metabolism that they consider to be specific features of malignant cells.[11]

Summary

Examples have been presented of the utility of drug-resistant microorganisms in secondary screening evaluations of new anticancer agents, first suggested for further study on the basis of primary screening results. We feel that such secondary screening data will be of value in selecting the most promising new agents for intensive further study.

Our knowledge about the mode of action of known anticancer agents has been increased significantly by the results of studies with drug-resistant microorganisms.

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Discussion of the Paper

Question: I wonder if 8-azaguanine ribotides or ribosides were ever tried on resistant cells. It seems that such an attempt would be the answer to 8-azaguanine resistance, providing the block in conversion is the reason for resistance.

Schabel: I do not know whether they have ever been tried, but I know these possibilities are under very active consideration. Brockman gave me these data, I think, because they are relative to drug-resistant microorganisms, but I am not familiar with all of the ramifications and details of other things that have been done. Hutchison is, however. Perhaps she can answer your question.

Dorris J. Hutchison (*Sloan-Kettering Institute for Cancer Research, and Sloan-Kettering Division, Cornell University Medical College, New York, N. Y.*): We have done extensive studies with 8-azaguanosine; the organisms resistant to 8-azaguanine are also resistant to 8-azaguanosine. At the moment we are trying to obtain the material for studying 8-azaguanylic acid.

Question: Is the mercaptopurine-resistant organism cross-resistant with the azaguanine-resistant organism?

Schabel: I believe it is.

Dorris J. Hutchison: Schabel has presented a number of interesting observations on the use of drug-resistant mutants of *Escherichia coli* and *Streptococcus faecalis* in screening for a "type" agent and for the study of drug action. Would the same results have been obtained if other mutants with identical resistance had been used?

Since some of the *S. faecalis* cultures just discussed were obtained from our laboratory, additional data relevant to these organisms, as well as some comparative studies on other mutants of similar history, seem to be in order. A brief description and the nomenclature of these mutants are presented in table 1.*

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* Maintenance media and other details may be found in *Cancer Research*, vol. 18, p. 214, 1958.

TABLE 1

STREPTOCOCCUS FAECALIS CULTURES

SF/0	*S. faecalis* ATCC 8043 (Parent strain)
SF/A	Resistant to A-Methopterin
SF/A_{kt}	Resistant to A-Methopterin
SF/A_k	Resistant to A-Methopterin
SF/A/0	Resistant to A-Methopterin
SF/A/MP	Resistant to A-Methopterin and 6-mercaptopurine
SF/MP/A	Resistant to 6-mercaptopurine and A-Methopterin
SF/MP	Resistant to 6-mercaptopurine
SF/MP_{cc}	Resistant to 6-mercaptopurine
SF/DAP	Resistant to 2,6-diaminopurine
SF/AZAG	Resistant to 8-azaguanine
SF/8-AZA	Resistant to 8-azaguanine

TABLE 2

QUANTITATIVE FOLIC ACID REQUIREMENTS (mγ/ml. FOR HALF MAXIMUM GROWTH)

S. faecalis	Medium		
	F-PP	F-PP + T	F
0	1.0	0.5	0.3
A	5.0*	5.0*	0.1
A_{kt}	†	0.3	†
A_k	0.5	0.5	0.5
A/0	0.25	0.25	0.25
A/MP	5.0*	2.0*	0.25
MP/A	1.0	1.0	0.3
MP	1.0	1.0	0.25
MP_{cc}	0.4	0.4	0.4
DAP	0.5	0.5	0.5
AZAG	0.3	0.3	0.3
8-AZA	0.3	0.3	0.3

Symbols: F-PP medium = glucose, amino acids, salts, and vitamins; F-PP + T medium = F-PP supplemented with 1 γ/ml. thymine; F medium = F-PP supplemented with adenine, guanine, xanthine, and uracil.

* Incubated for 48 hours. All other incubations were for 22 hours.

† Requires thymine.

The requirement by *S. faecalis* ATCC 8043 of folic acid for the synthesis of both purines and thymine in certain media has made it a most useful and versatile organism for the study of folic acid, purine, and pyrimidine antagonists. In TABLE 2 the folic acid (PGA) requirements of a number of *S. faecalis* cultures are compared in three media; several alterations have been observed. If first a comparison is made among the organisms resistant to A-Methopterin, it will be seen that a unique pattern does not exist. SF/A is extremely inefficient in purine synthesis; SF/A_{kt} has lost completely the capacity to synthesize thymine; while SF/A_k is more efficient than the parent in the synthesis of purines and thymine. SF/A/0 is more efficient than SF/A_k; SF/A/MP is similar to SF/A; SF/MP/A is different from all the others in that thymine supplementation of the F-PP medium does not alter the folic acid requirement; while purine supplementation of the medium lowers the quantitative folic acid requirement.

TABLE 3

GROWTH RESPONSE OF *S. FAECALIS* TO PURINE DERIVATIVES

Substrate concentration 3×10^{-4} M	Optical density*						
	0†	MP	MP$_{cc}$	A/MP	AZAG	8-AZA	A$_{kt}$‡
Adenine	0.37	*0*	0.41	0.48	*0*	*0*	0.11
Adenosine	0.36	*0*	0.45	0.48	*0*	*0*	0.34
Adenylic acid-2′	0.03	0	0.03	0	0.07	0.03	0.07
Adenylic acid-3′	0.08	*0*	0.07	0.10	0.09	0.09	*0.29*
Adenylic acid-5′	0.03	0	0	0	0.07	0.09	*0.36*
Guanine	0.16	*0*	0.41	0.51	*0*	*0*	0.45
Guanosine	0.16	*0*	0.49	0.51	*0*	*0*	0.45
Hypoxanthine	0.38	*0*	0.41	0.48	*0*	*0*	0.44
Inosine	0.34	*0*	0.45	0.48	*0*	*0*	0.43
Inosinic acid	0	0	0	0	*0.13*	*0.10*	0.03
Xanthine	0.43	0.54	0.44	0.42	0.41	*0*	0.44
Xanthosine	0.25	0.49	0.44	0.45	0.46	*0*	0.43
2,6-Diaminopurine	0	0	0	0	0	0	0.06
6-Mercaptopurine	0.15	*0.03*	*0.03*	*0.35*	*0*	*0*	*0*
8-Azaguanine	0	0	—	0	0	0	—
Isoguanine	0.15	0.15	0.14	0.18	—	—	0.12

* Italic figures indicate the major differences in the mutants in comparison with SF/0.
† SF/A, SF/DAP, SF/MP/A, and SF/A/0 have a similar pattern.
‡ SF/A$_{k}$ has a similar pattern.

Two mutants resistant to 6-mercaptopurine (SF/MP and SF/MP$_{cc}$) also are different in the folic acid requirements. SF/MP is identical with SF/MP/A, while SF/MP$_{cc}$ is qualitatively similar to SF/A/0. Three other purine analogue-resistant mutants (SF/DAP, SF/AZAG, SF/8-AZA) are similar in that they, like SF/MP$_{cc}$, are efficient in the *de novo* synthesis of both purines and thymine.

Further comparative studies with these organisms have been made in regard to their growth on a variety of purine derivatives. Certainly the quantitative differences observed in the folic acid studies could be used to group the mutants in regard to their capacity to synthesize purines and thymine, but this does not give a complete picture of the qualitative alterations. The results presented in TABLE 3 show the changes in the capacities of the organisms to grow on purine derivatives in the absence of folic acid. With SF/0 as the control, it has been observed that SF/A, SF/DAP, SF/MP/A, and SF/A/0 have identical responses to purine derivatives, while SF/MP grows only on xanthine and xanthosine and not on 6-MP; SF/MP$_{cc}$ is similar to SF/0, although it does not use 6-MP as a purine source. SF/A/MP grows almost as well on 6-MP as on adenine or any other natural purine. Note that SF/AZAG is similar to SF/MP in the utilization of xanthine and xanthosine, and also grows slightly on inosinic acid and the adenylic acids. SF/8-AZA is comparable to SF/AZAG, although it has lost the ability to use the xanthine-containing compounds. SF/A$_{kt}$ and SF/A$_{k}$ are similar to SF/0, but they have the additional capacity to grow on adenylic acid-3′ and adenylic acid-5′.

The multiple alterations observed among these mutants suggest that resistance to an antimetabolite does not result in a unique pattern of metabolism.

The numerous differences observed make them useful tools for the study of the mode of action of growth inhibitors; furthermore, they are potential candidates for secondary screening systems. These mutants of *S. faecalis* indeed suggest that in nature there are many alternate pathways that can be followed to render organisms resistant to once-potent inhibitors.

Comment: With respect to the 8-azaguanylic acid, we have tested it on a sensitive strain of L 1210 and have found that the nucleotide is no more effective than the free base. This can be explained readily by the fact that the nucleotide probably is broken down, either to the nucleoside or to the free base; hence, the carcinostatic potency is no better than that of the free base. I should not be surprised if, in the case of the resistant strain, the same problem did not exist. Therefore, this method of proving the mechanism of resistance is very difficult.

Comment: It might be interesting to compare some of our results with *Lactobacillus casei* drug-resistant strains with Hutchison's, because there are some interesting differences. The MP-resistant *L. casei*, for example, is a rather stable mutant in the sense that it is always obtained with the same type of metabolism if always isolated in the same way. In this it differs from *Streptococcus faecalis*. Furthermore, this mutant is not cross-resistant to azaguanine, nor is the azaguanine-resistant *L. casei* cross-resistant to 6-mercaptopurine. When we study the growth requirements of these two strains, the reason becomes obvious; namely, that the mercaptopurine-resistant strain will not grow on either hypoxanthine or adenine. The azaguanine-resistant strain will grow on adenine. The 6-mercaptopurine-resistant strain will grow on guanine. Thus, there is a very great difference in the mechanism of resistance for these two strains.

The question Mandel raised as to whether the azaguanylic is transformed to azaguanine might be turned around to read, "Is the azaguanine transformed to asaguanylic?" The test on the resistant strain would be the point in question. If the L 1210-resistant strain were inhibited by azaguanylic, we might assume that the azaguanylic and azaguanine were of equal activity, because the azaguanine was transformed to azaguanylic, and not vice versa.

GENERAL CONSIDERATIONS IN THE USE OF MICROORGANISMS IN SCREENING ANTITUMOR AGENTS*

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The prophet's mantel is easily transformed into a strait jacket or dunce cap. As microbiologists rather detached from clinical urgencies, we have cause to shy away from pronouncements. However, with a rashness incited by gratifications in providing microbial research tools for such scientists as vitamin biochemists and photobiological physicists, we shall advance some notions.

Chemists can create compounds faster than biologists can test them—unless screening procedures become more effective. Two kinds of nets are necessary in an effective screening procedure: (1) one sufficiently wide to detect all classes of cytotoxic compounds, and (2) one sufficiently fine meshed to guide the choice of clinically useful compounds within a particular class of cytotoxic agents. There is no compelling reason for the coarse and the fine-meshed nets to use the same microorganisms.

Initial Screening for Toxicity; Phagotrophic Protozoa

Cancer being a disease of cellular, that is, multicellular organisms, those chosen to detect cytotoxic agents, in general, should simulate metazoa metabolically. Ideally, each differentiated metazoan cell line should be represented in the screen—a present impossibility since, aside from expense, the rearing of pure lines of specialized cells is in its infancy. The practical course for detecting general toxicity is to employ hardy yet sensitive microorganisms easily grown in chemically defined media. Thanks to their exploitation for microbiological assay of vitamins and amino acids, such easily handled bacteria as *Lactobacillus casei* and *Streptococcus faecalis* are available. With the wisdom of hindsight we see that the dependence of these bacteria, under well-understood conditions, on such attractive chemotherapeutic targets as folic acid, purines, and pyrimidines, contributed to their successful use.

Bacteria are phylogenetically remote from us, however. The achievements with bacteria, while a dramatic testimony to the unity of biochemistry, should spur the hunt for more realistic screening organisms. For a time, we think, these are to be found among protists with a conventional morphologic nucleus. Repeatedly we have urged the use of particle-ingesting protozoa as

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† Also Department of Chemistry, Mt. Sinai Hospital, New York, N. Y. Portions of this paper are from a dissertation presented by H. Baker in partial fulfillment of the requirements for the degree of Ph.D. in Biology at New York University, New York, N. Y.

‡ Portions of this paper are from a dissertation to be presented by S. Scher in partial fulfillment of the requirements for the degree of Ph.D. in Botany at Rutgers University, New Brunswick, N. J.

objects of biochemical research, in part because of their exceptional ability to utilize high-molecular and fat-soluble materials, and we have sought to domesticate new kinds of phagotrophs. The task afterward is to ascertain whether such a screen consisting mainly of phagotrophs catches all cytotoxic compounds. In this initial stage we are using three phagotrophs: the brown-pigmented *Ochromonas malhamensis*, *Tetrahymena pyriformis*, and a small soil amoeba, *Hartmannella rhysodes*. These protozoa may be as phylogenetically remote from each other as they are from metazoa. We are also testing two nonphagotrophs: *Crithidia fasciculata* (a nonpathogenic member of the Trypanosomidae) and *Euglena gracilis z*. These may be phylogenetically remote from the phagotrophs. *Euglena* is clearly related to the green algae and, hence, to the land plants and probably to the fungi; screening with *Euglena* might, then, catch much the same compounds as would fungi.

The idea that protozoa are much closer to metazoa than are the bacteria is not suggested entirely by macromorphology; protozoal behavior vis-à-vis antibiotics emphasizes the metabolic gulf between them and the bacteria and, in contrast, their kinship to metazoa. The examples of penicillin, chloramphenicol, streptomycin, the tetracyclines, neomycin, and many others emphasize the fact that it is not very difficult to find potent antibacterial antibiotics with little toxicity to metazoa. It seems much harder to find antiprotozoal agents with low toxicity toward the host; when such agents are found, they are apt, as with pyrimethamine and puromycin, to have some antitumor properties and a narrow margin of therapeutic safety.

Should the present phagotroph trio prove unsatisfactory, we look upon this as a back-to-the-old-drawing-board situation. Many more phagotrophs abound in nature. The phagotrophic marine dinoflagellate, *Oxyrrhis marina*, is available in pure culture. From its finicky food habits and predilection for lipid-containing particles, its nutritional requirements may rival the complexity of vertebrate cell cultures; therefore, a great deal of work will be required to standardize its cultivation. The amoebae are a vast and polyphyletic group. It is a simple matter to obtain pure cultures: amoebae frequently come up, for example, on antibiotic-containing serum-sea water agar; they can be fed on pure cultures of live food, such as a streak of diatoms on agar. There is no reason to think that the marine amoebae are closely related to the soil forms, or that small amoebae have any special kinship to the familiar large amoebae. Then there are the very different foraminifera and heliozoa—again the initial problem is to replace live food with nonliving materials. A voracious euglenoid, *Peranema*, is available in an almost completely defined medium. Finally, a large assortment of nonphagotrophic Trypanosomidae are available in pure culture, representing many different patterns of sensitivity to chemotherapeutic agents.

Symbionts and Tissue Cultures

Suppose that protozoa, including those under "development" or still in the dream department, do not work out as initial screening organisms and, worse, that no other protists show outstanding worth. A logical course would be to resort to metazoa—but which? And should one use intact metazoans or cell lines from them? The choice must take into account phylogenetic and practical considerations. In protists we deal, in the jargon of biochemists, with "systems" capable, as are cancer cells, of unlimited growth. We adhere to Osgood's[1] view that the ineluctable problem of cancer research is to identify the inhibitors of cell division produced by differentiated cells; embryologists have long grappled with the exceedingly difficult corollary problem of differentiation factors. The artificial administration of such inhibitors or of differentiation factors should provide powerful therapies; indeed, Osgood points out that vitamin B_{12}, in permitting the normal differentiation of the erythrocyte, corrects in pernicious anemia a condition almost indistinguishable from a conventional malignant process. How, then, to get on with the job of identifying these inhibitors and differentiation factors? We think, for reasons to follow, that some of the obstacles may be by-passed by the development of artificial symbiotic systems with cell lines of lower metazoa as one set of partners.

Multicellular animals began as coelenterates or acoelate flatworms.[2] The nutritional requirements and interactions of cell lines of these lowly metazoa might reveal much about the hormonal controls and differentiation factors that permit these cell lines to form the harmonious individual. Most of these factors—including the most fundamental ones—hardly seem accessible yet in vertebrates; perhaps lower metazoa offer better experimental avenues. An assortment of cell lines, comprising most or all the types needed to reconstitute the individual and presumably numbering far fewer cell types than required to make a good start at reconstituting a vertebrate, might provide an effective screen while, at the same time, opening the way to identifying the almost completely unknown array of growth regulators. These are enterprises we must not neglect while deploying our main resources toward problems of growth regulation in vertebrates. That these cell lines will be any easier to cultivate than are vertebrate cells seems too much to hope for, and we do not know that such a situation would be desirable. How else would new growth factors or morphogenetic factors be brought to light? "Lower" metazoa have had just as much evolutionary time as we have had to develop complex nutritional requirements. It is a reasonable expectation that the common denominator of the nutritional requirements of these cell lines is the requirements of the intact organism—as demonstrated for vertebrate cells. Knowledge of the nutritional requirements of lower metazoa thus emerges as a serious limiting factor in long-range cancer research; the present position will be surveyed in a forthcoming publication of The New York Academy of Sciences, *Axenic Culture of Invertebrate Metazoa: a Goal.*

What if the nutritional requirements of cell lines of lower invertebrates do prove to be as complicated as those of vertebrates? One need is a drastic simplification and standardization of vertebrate cell culture technique. Thus Evans[3] says, ". . . to aid investigators and to save them time, it would be a good idea to send out with a given strain of cells the medium devised for the strain of cells."

Complaints about the ever-heavier burden of expense and training imposed by modern cell- and tissue-culture techniques are being voiced more and more freely. For example, "... we see a huge list of purified chemicals, enzyme substances, coenzymes—materials which are very expensive and which are often hard to obtain. Once the nutritional requirements of cells have been worked out, it will be up to us to go back again and try to find cheaper sources of all these materials ... when we work with hundreds of liters we cannot use purified coenzyme A or some of the amino acids. Also, I would be pleased to see more effort made to reduce the number of ingredients ...."[4] Such pleas may understate the need. If we are to produce inhibitors and differentiation factors on a practical scale, they will be too complicated to be made by artificial synthesis; they must be produced by the cell lines themselves, by large-scale techniques resembling the production of viruses, as in making Salk vaccine or, to take a simpler example, as B_{12} is produced industrially by bacterial synthesis. Coenzyme A is best prepared from yeast; the obstacles to preparation of a pure preparation—as yet unavailable—recently have been detailed.[5] Here, in effect, microbes already are being used to supply otherwise poorly accessible nutrients. There is also a desperate need for cell and organ cultures to provide spare parts; for example, it is quite likely that spleen or bone marrow cultures will be needed to replace leukemia- or radiation-destroyed hematopoietic cells.

The sought-for drastic simplification in using cell lines for screening may come from the use of algal symbionts, much as "feeder" cells are used in cell culture. As long ago as 1934[6] *Chlorella* was used as an artificial symbiont, but tissue culturists and algologists of that time showed little interest. The specifications for a suitable symbiont are now being framed. A symbiont should (1) grow well at tissue culture temperature, say 34 to 37° C.; (2) tolerate the cell culture media; (3) not grow in the dark, at least not in the cell culture medium; and (4) be nonallergenic.

Until recently, algologists knew little about growing algae at mammalian body temperature. Furthermore, little was known about algae unable to grow in the dark ("obligate phototrophs"). Even less was known about the cultivation of hardy brackish-water algae, the algae one would expect to withstand best the salts, osmotic pressure, and high organic content of tissue culture media. The idea, in short, is to convert two-dimensional cell cultures to three-dimensional balanced aquaria, with the ratio of metazoan cell to alga controlled simply by varying the level of illumination.

Two of our colleagues, P. A. Zahl and J. J. A. McLaughlin, are seeking to fuse algology and cell culture; they are studying the algal symbionts of marine invertebrates, keeping in mind the possibility that the best artificial symbionts may not originally be symbionts at all but, rather, free-living thermophilic algae. A detailed paper in preparation[7] follows up the earlier report[8] on the artificial cultivation of these "zooxanthellae." Zooxanthellae are proving to be predominantly, if not exclusively, dinoflagellates, which adds interest to the use of dinoflagellates as screening organisms. With symbiont-containing cell cultures, one must learn to distinguish between agents acting on the cell and agents acting on the symbiont.

A problem before us, then, is to screen algae for their use as symbionts in assembling symbiont-cell culture pairs, these in turn to be used as screens for cytotoxic factors and for more subtle growth regulators. In addition to supplying oxygen and removing CO_2 along with, perhaps, lactate, a superior feeder-alga might be one liberating good quantities of unstable and therefore troublesome nutrients such as ascorbic acid, vitamin E, coenzyme A, and cocarboxylase. The excretion by algae of organic materials is becoming a popular subject of investigation now that water organisms, especially phytoplanktons, have been shown to be intricately interdependent in respect to organic as well as inorganic nutrients (see review by Provasoli[9]). The main obstacle to practical examination of algae for excretion of nutrients of value to cell culture is microbiological: the absence of microbiological assays for many important nutrients, particularly the aforementioned fat-soluble ones. This situation, and possible remedies in the shape of improved techniques for screening for nutritional mutants in algae, protozoa, and fungi is one discussed in the increasingly overlapping literature on chemical microbial genetics and the mode of action of antibiotics and other chemotherapeutic agents.

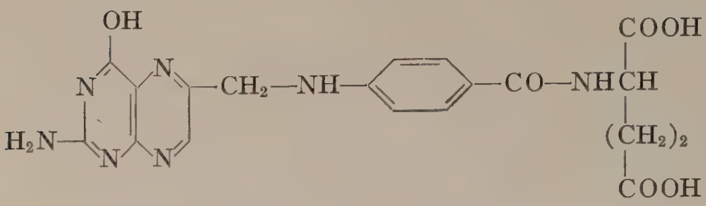
"Systems" for Antimetabolites

We have been discussing detection of cytotoxicity, that is, initial screening. Once the mode of action of a cytotoxic agent is suspected or known, the next step is to chart the antimetabolite activity of congeners of the compound; Woolley has discussed this procedure.[10] A working assumption at this stage is that ineffective antimetabolites in microbial systems probably will be ineffective against metazoan cells or intact metazoa (exceptions are known, notably among antimalarials). For antimetabolite tests, it is informative to use a pair of closely related protists, one exogenously requiring the target metabolite, the other synthesizing it. This may bring into the open differences in susceptibility that reflect differences in accessibility and rate of synthesis of the target metabolite. If an easily handled microorganism has a clearly defined or absolute exogenous requirement for a metabolite and, if this requirement has been sufficiently studied, the microorganism is likely to have been described as an assay organism. Therefore, in picking microorganisms for antimetabolite studies, one might well consult the roster of recommended assay microorganisms. Microbiologically assayable metabolites, along with brief descriptions of especially useful assay organisms, have been listed[11] and need not be repeated; instead, a few systems of immediate interest in cancer research will be mentioned here. Uses of lactobacilli, *Tetrahymena*, and yeasts in testing for antimetabolite activity, especially antifolic, antipurine, and antipyrimidine activity, are treated in several other papers elsewhere in this monograph.

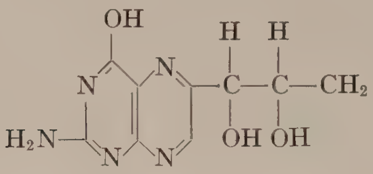
Crithidia Factor and Biopterin

Development of resistance to antifolics is so grave a problem that any lead to a possible solution is to be pursued tenaciously. The flagellate *Crithidia fasciculata* may have here a unique role—adding to its value as a screening organism. Some strains of *C. fasciculata* need a growth factor; one of the active compounds in urine, biopterin, has been isolated and synthesized.[12]

C. fasciculata has an independent requirement for folic acid.[13] High con-



Folic acid



Crithidia factor

centrations of folic acid mask the need for *Crithidia* factor. Folic acid may then be a precursor of *Crithidia* factor. Inspection of the structural formulas of biopterin and folic acid shows that mere cleavage of folic acid at the C^9—N^{10} link will not yield *Crithidia*-active compounds; condensation with a carbohydrate or an amino acid such as threonine may be necessary. Enzymes that cleave folic acid and Aminopterin at the C^9—N^{10} link have been described for microorganisms[14] and blood.[15] In *Candida tropicalis* the enzyme is adaptive—growth appears after a lag—and an intensely yellow pigment accumulates, which behaves as a free pteridine.[16] *C. tropicalis* could not overcome inhibition by 3′,5′-dichloroaminopterin. Kidder[16] found *Tetrahymena* susceptible to 3′,5′-dichloro derivatives of Aminopterin. Are some antifolics more apt than others to induce or activate folic-splitting enzymes in blood cells? Does a splitting enzyme control a step in the synthesis of essential unconjugated pteridines? Do some halogenated antifolics owe their prolonged action to the fact that they do *not* induce development of splitting enzymes? One wonders whether this situation parallels that summarized by Lwoff[17] for β-galactosidase in *Escherichia coli*, where methyl-β-D-galactoside, which is not itself susceptible to the enzyme, is nevertheless an inducer. If induction phenomena do play a part, then the clinical value of an antifolic cannot be gauged by short-term microbial tests. If (1) the folic-splitting enzyme functions in the synthesis of *Crithidia* factor, and (2) folic splitting is the rate-limiting reaction in synthesis of *Crithidia* factor, then estimation of *Crithidia* factor in blood or urine in patients undergoing antifolic therapy might provide a realistic means of gauging the effectiveness of antifolics. The assay has been simplified,[18] and examinations of clinical situations are under way.

There is evidence[19] that some antifolics have anti-*Crithidia* factor activity as well. Since we do not know what these unconjugated pteridines do, it is premature to assess the possibilities of sequential blocking along the folic → *Crithidia* factor pathway.

The behavior of xanthopterin, a 6-hydroxy unconjugated pteridine, counters the idea expressed earlier that differentiation factors may be too complicated for artificial synthesis. Haddow[20] reported that the kidneys of rats injected with xanthopterin undergo speedy hypertrophy because of a great outburst of mitotic activity in the kidney tubules. Xanthopterin is inert for *C. fasciculata*; no microbiological assay for xanthopterin-type unconjugated pteridines is known. Xanthopterin, further, may be an artifact, not an essential product of folic acid metabolism.[21] The possibilities of sequential blocking within the folic pathway are, then, wider than was first thought but, as noted, knowledge of these new targets is meager.

Vitamin B_{12}. This doubly heterocyclic compound is an obvious chemotherapeutic target. The intact molecule may be modified to yield anti-B_{12}'s,[22] but this gambit appears to offer fewer practical possibilities than does tinkering with the dimethylbenzimidazole moiety. Some substituted benzimidazoles and related heterocycles in preliminary reports act as anti-B_{12}'s for *E. coli*.[23-25]

For studies of B_{12} antagonists, our main experimental object is *Ochromonas malhamensis*, whose pattern of B_{12} requirement seems identical with man's. To test whether this identity is fortuitous, we are trying to find why its B_{12} requirement increases steeply with temperature;[26] should this "thermal" B_{12} requirement have the same metabolic origin as the enhanced B_{12} requirement of hyperthyroid vertebrates, we should have more reason for looking upon *O. malhamensis* as "humanoid." This issue is not trivial; if microorganisms do exist with metabolic patterns closer to the metazoa than are the microorganisms hitherto used, research on mammalian metabolism would be accelerated. The responses of *O. malhamensis* to antiprotozoal agents suggest at this time that its behavior mimics the host rather than such parasites as hemoflagellates and the malarial organism.

In using *O. malhamensis* as a primary detector of toxicity, we are taking advantage of its ability to grow through a wide *p*H range, roughly 4.0 to 7.8; we are testing ionizable compounds at *p*H 4.8 and 7.2 to detect differences in effectiveness due to permeability.

Another chrysomonad, *Ochromonas danica*, does not need exogenous B_{12}.[27] It grows vigorously, but since its nutritional preferences differ from those of *O. malhamensis* in respects as yet imperfectly known, it is still in the development stage. The ultrasensitive B_{12} requirement of the rather permeable *z* strain of *Euglena gracilis* provides another means of detecting anti-B_{12} activity.

Detection of Toxic Side Actions: Dinitrophenol and Menadione

We have been worrying about the masking of valuable specific toxicities by nonspecific toxicities, a likelihood, since molecules have several reactive groupings. One kind of toxicity, interference with oxidative phosphorylation, is a property of widely diverse compounds such as 2,4-dinitrophenol, hexachlorophene, azide, barbiturates, salicylates, gramicidin, tetracyclines, 2,4-dichlorphenoxyacetate, and quinacrine.[28] Clearly, a simple means of detecting and overcoming (or at least allowing for) this mode of toxicity is desirable. To start, we are studying reversal of dinitrophenol (DNP) inhibition of the growth of *O. malhamensis*. As seen in TABLE 1, DNP progressively impairs the utilization of PO_4, which is hardly unexpected in light of the host of papers on the upsetting by DNP of PO_4-uptake and function in enzyme

TABLE 1

REVERSAL OF DINITROPHENOL (DNP) BY PHOSPHATE IN *OCHROMONAS**

KH_2PO_4 (mg. %)	Dinitrophenol			
	0	0.1 mg. %	0.2 mg. %	0.4 mg. %
0	0.24	0.09	0.02	0
0.1	0.68	0.54	0.35	0
0.3	1.32	1.12	0.6	0
1.0	2.0	1.9	1.0	0
3.0	2.5	2.5	0.6	0
10	2.6	2.6	1.1	0
30	2.6	2.6	1.7	0
100	2.6	2.6	2.2	0

* Medium with 0.3 per cent L-glutamic acid; growth expressed as optical density.

preparations. However, no paper on overcoming DNP inhibition in intact microorganisms has come to our attention. In the one paper on metazoa (rat),[29] overcoming the inhibition of growth by DNP required the administration of insulin + growth hormone + glucose. In the presence of excess PO_4, we find the inhibition of growth in *O. malhamensis* and *Euglena* by DNP to be reversed competitively by L-glutamic acid. This second action is in line with the report that DNP has the configuration of inhibitors of L-glutamic acid dehydrogenase, most clearly shown by 5-bromo- or 5-nitrofuroic acid.[30] Future work may show that oxidative phosphorylation in some tumors is more vulnerable than normal tissue to some uncoupling agents.

With DNP exemplifying a small molecule exerting at least two distinct actions, both detectable and overcome by simple nutritional means, we examined another metabolically reactive aromatic compound, menadione (2-methyl-1,4-naphthoquinone), a widely used form of vitamin K, reported to be occasionally toxic for infants.[31] We confirmed Schopfer's[32] finding that nicotinic acid acts as an antagonist. As is common in such cases, however, this competitive reversal holds only through a limited concentration range; then something else comes into play, as shown in TABLE 2 by the effectiveness of the nicotinic acid–proteose-peptone combination. Much of the same thing

TABLE 2

REVERSAL OF MENADIONE INHIBITION OF *OCHROMONAS MALHAMENSIS*

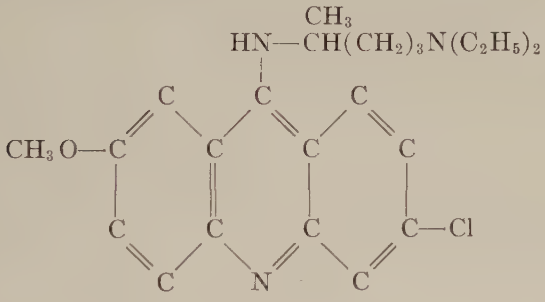
Menadione (mg. %)	No add.	Nicotinic acid (2.0 mg. %)	C.S. No. 9 * (5.0 ml./100)	Proteose-peptone (0.4%)	Nicotinic acid + proteose-peptone	Nicotinic acid + proteose-peptone + C.S. No. 9
0	10	10	10	10	10	10
0.04	2.2	10	10	10	10	10
0.08	0	7.7	10	10	10	10
0.15	0	2.6	1.2	10	10	10
0.2	0	0	0	0.95	10	6.0
0.3	0	0	0	0	0	0

* "C.S. No. 9" = "complete supplement," a shotgun mixture of vitamins, amino acids, purines, and pyrimidines; details of its composition and of the basal medium have been given elsewhere.[26]

had been observed for DNP; there is a point beyond which toxicity is no longer reversed by phosphate + glutamic acid. The value of identifying the metabolites that extend the range of reversible inhibition had been shown strikingly in Shive's[33] isolation of vitamin B_{12} as an annuller of sulfanilamide inhibition of *E. coli*.

Among cytotoxic heterocycles, the benzimidazoles appear to provide outstandingly good materials for charting the groupings that make for activity against purines, pyrimidines, folic acid, riboflavin, heme, *Crithidia* factor, and B_{12}. Benzimidazole itself has a poorly understood toxicity; clean-cut reversal by purines is seldom observed. For example, purines did not annul inhibition of streptolysin production by hemolytic streptococci.[34] As benzimidazoles are being put to such diverse uses as blocking amino acid incorporation in staphylococci (5,6-dimethylbenzimidazole)[35] and blocking the development of *Rana pipiens* (methoxynitrobenzimidazoles and related heterocycles),[36] the need grows for systematic studies of the benzimidazoles. Other benzimidazoles inhibit egg cultures of influenza virus.[37]

Knowledge of the mode of toxicity of many other important complex heterocycles is as unsatisfying as knowledge of the benzimidazoles. Consider again the well-known antimalarial quinacrine (Atabrine, mepacrine).



Quinacrine (Atabrine)

It interferes with flavin enzymes,[38] is presumably displaced by polyamines from inhibitory combination with bacteria[39] and, from spatial considerations, is thought to interfere with adenylic acid.[40] Its toxicity to *Streptococcus faecalis* is annulled by folic acid.[41] Which action is decisive for antimalarial activity—or is it none of these? Problems such as this occur in elucidating relationships between antiviral and antitumor chemotherapy. Even compounds that might be expected to act unequivocally against nucleic acids may have other actions. The mitotic inhibition caused by 6-mercaptopurine in tissue cultures of Sarcoma 180 and embryonic mouse skin was most effectively blocked by coenzyme A.[42] Burk[43] goes so far as to assert that the so-called antipurines and antifolics primarily cripple the hexokinase reaction of mitochondria—that these inhibitors are to be looked upon as anti-ATP's.

Antiviral and Antitumor Chemotherapy

Chemotherapeutically, the smaller viruses resemble tumors: both live in chemical intimacy with the host; the most consistent differential inhibitions come from fraudulent building blocks apt to yield crippled nucleic acids. Indeed, the review on chemotherapy of viruses by Matthews and Smith[44] necessarily outlines the biosynthesis of nucleic acids and, were designation of one or another transplantable tumor substituted in much of the text for the viruses in question, one would hardly notice the substitution. The practical problems take the same form: the *in vivo* ineffectiveness of many compounds after *in vitro* results had raised hopes, and the diminishing returns in screening once one departs from obvious interference with nucleic acid metabolism or of catalysts for nucleic acid synthesis. Quinacrine has been reported to protect mice against Eastern equine encephalomyelitis virus.[45] Again, which if any of the aforementioned actions of Atabrine operate?

Present knowledge must be looked upon as merely a crude beginning with isolated forays into specific activities. Gottlieb[46] reviews general factors in the action of antimicrobial agents. There is an uncomfortable truth in the gibe repeated by Burk[43] that today we have an "alchemotherapy," not chemotherapy.

Common problems—and, let us hope, common answers—emerge in dealing with intracellular parasites that, like tumors, insinuate themselves into the intimate chemical machinery of the host. As biological research engages a greater share of the free energy of society, a closer coordination of microbiological-oncological investigations will be an economic as well as scientific necessity. Malarial parasites and the hardly touched thousands of other sporozoa must be outwitted. Flagellates such as *Trypanosoma cruzi* and the leishmanias, having learned to live intracellularly, are evolving toward virushood. Anaplasmas and babesias are further along the same path. Such tasks and problems converge on the virological. Likewise, bacteriologists dealing with rickettsiae and the multitudinous bacterial intracellular symbionts of arthropods and parasitologists dealing with blood flukes and microfilariae also approach the functions of virologists. Finally, to mark the common ground, there is the recognition that many tumors are virus induced (or at least induced by filterable agents), and that some tumors may be destroyed by viruses. It follows that future screens for anticancer agents will make greater use of viruses and of highly evolved parasites. To return to problems of everyday practicality: there will be a development of cheap, easily reproducible culture media for tissue culture—one key to growing fastidious parasites as well as new lines of differentiated cells. More attention must be paid to such mundane matters as the cost of culture media and improvement of mailability by formulating them as far as possible as dry mixes. There will be continued examination of diverse free-living and parasitic organisms to find the ones best suited to mode-of-action studies, coupled with an intensified hunt for new growth factors to serve as chemotherapeutic targets. This will entail more studies of fastidious parasites and fastidious cell lines.

We glimpse, then, that research on screening procedures is another aspect of the problem of the nature of cancer itself; that amid the drudgery and pressures of screening compounds we must allot some resources for improving the procedure itself and for furthering enterprises aimed at erecting new chemotherapeutic targets. We are hopeful; the demolition of the man-made barriers between morphologic macrobiology, microbiology, evolution, genetics, and chemistry, as reflected, for example, in a recent text,[47] portends that researchers will not hesitate to attack the cancer problem with the full arsenal of microbiology and chemistry.

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SPECIAL MICROBIOLOGICAL SYSTEMS

I. ANAEROBIC ORGANISMS*

By William T. Bradner

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For the past two years my associates and I have been studying anaerobic bacteria as possible screening tools for antitumor agents.[1] This investigation was undertaken on the premise that the anaerobes might respond more like tumor tissue than do aerobic organisms because of the greater similarity of their metabolism to that of tumors. The observation by Coffey and his co-workers[2] in 1954 that 6/6 clostridia were markedly inhibited by azaserine seemed to support such a theory.†

Since anaerobic organisms are more cumbersome to cultivate than aerobes, it was considered of primary importance to determine whether or not in some area of cancer research they could offer information superior to that gained with aerobes to a degree that would justify the increased complexity involved in their use. Although in the course of the study some interesting and perhaps unique inhibitory responses have been noted, it is difficult to ascertain whether anaerobic requirements were responsible for the results, or whether the responses only represented the normal expectation for a group of bacteria not previously examined. This report is confined strictly to a study of inhibitory responses by the anaerobes as a basic screening procedure. Our findings are based on a comparison of the results obtained with agents of interest in cancer research with the inhibition of *in vivo* tumor growth and animal toxicity.

Results

Ten nonpathogenic organisms representing a variety of genera were selected on the basis of their ability to grow well in common laboratory media (TABLE 1). The agar plate penassay disk procedure was used, and inhibition was noted at 3 dilutions: 1000, 100, and 10 μg. total concentration per disk.

A previous communication[3] has reported results obtained with a number of materials including, specifically, the 27 compounds used in the American Cancer Society (ACS) "Investigation of Diverse Systems for Cancer Chemotherapy Screening."[5] In this way correlations were made with tumor tests. Shown in TABLE 2 for purposes of illustration are the first 10 compounds of the ACS list and the activity rating of each against the tumor systems, the non-tumor systems, the anaerobes, and one particular organism, *Clostridium feseri*. By application of the ACS procedure of arbitrarily considering positive any compound that inhibited one third or more members of a system, the anaerobe results could be scored against the tumor responses for mismatched positive; that is, cases where the anaerobes were inhibited and the tumors were

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* The work reported in this paper was supported by a research grant from the National Cancer Institute, Public Health Service, Bethesda, Md.

† Marked sensitivity of several clostridial strains to azaserine has been confirmed independently in our laboratory[3] and in the laboratory of DiPaolo and Rosenfield.[4]

not (as, for example, with chloramphenicol), mismatched negative (for example, urethan), and total matching positive and negative results. Dividing the latter figure by the total tests yielded the so-called "matching score." The inhibitory results obtained with the 27 ACS compounds revealed *Cl. feseri* as the microorganism best correlated with the tumor systems.

This study now has been extended to 90 compounds, using for comparison a tumor system standard assembled from inhibitory results obtained with Sarcoma 180 and Adenocarcinoma 755 in our laboratory. The establishment of a standard of antitumor activity for these compounds presented the problem of making an arbitrary decision as to which were active and which were inactive. This was resolved simply by rating as positive each compound that had demonstrated inhibitory activity by the usual criteria employed against either tumor at any time. Likewise, compounds giving marked inhibition of any one bacterial species or slight inhibition of four tenths or more were considered active or positive with respect to the anaerobes. Although it can be argued

TABLE 1
BACTERIA USED FOR INHIBITION STUDIES UNDER ANAEROBIC CONDITIONS

Organism	Strain
Bacteroides limosus	ATCC* No. 8426
B. pseudoramosus	ATCC No. 8489
B. tumidus	ATCC No. 8496
Butyribacterium rettgeri	ATCC No. 10825
Clostridium acidi-urici	9A†
Cl. cylindrosporum	ATCC No. 7905
Cl. feseri	ATCC No. 10092
Micrococcus aerogenes	ATCC No. 9321
Propionibacterium freudenreichii	ATCC No. 6207
P. shermanii	ATCC No. 9617

* ATCC: American Type Culture Collection, Washington, D. C.

† Courtesy of H. A. Barker, University of California College of Agriculture, Davis, Calif.

TABLE 2
COMPARISON OF ANAEROBES WITH TUMOR AND NONTUMOR SYSTEMS*

Compound	Tumor* systems	Nontumor* systems	Anaerobic bacteria	*Clostridium feseri*
Nitrogen mustard	+†	+	+	+
A-Methopterin	+	+	−	+
Hydrocortisone	+	−	+	+
N-Methylformamide	+	−	−	+
N-Methylacetamide	+	−	−	+
6-Mercaptopurine	+	+	+	−
Urethan	+	−	−	+
Methylcarbamate	−	−	−	−
Chloramphenicol	−	+	+	+
Potassium arsenite	+	+	+	+

* Cancer Research, Supplement No. 3.

† + = Response by one third or more of the members of the system.

TABLE 3

COMPARISON OF ANAEROBE INHIBITION WITH THAT OF SARCOMA 180 AND CARCINOMA 755

	Total compounds	Mismatched		Matching negative and positive	Matching score (No. matching/No. compounds)
		Negative	Positive		
Bacteroides limosus	86	29	15	42	0.49
B. pseudoramosus	86	20	21	45	0.52
B. tumidus	83	20	15	48	0.58
Butyribacterium rettgeri	88	30	16	42	0.48
Clostridium acidi-urici	90	32	12	46	0.51
Cl. cylindrosporum	90	27	16	47	0.52
Cl. feseri	88	21	15	52	0.59
Micrococcus aerogenes	86	32	15	39	0.45
Propionibacterium freudenreichii	89	37	12	40	0.45
P. shermanii	90	28	11	51	0.51
Ten organisms	90	21	20	49	0.55
Three organisms*	90	10	21	59	0.66

* *B. pseudoramosus*, *Cl. feseri*, and *P. shermanii*.

that this may be an oversensitive standard, it seemed the most logical, since any observation of activity in a screening system becomes the subject of further investigation.

The results were not outstanding for either the 10 organisms considered as a group or for any of them considered individually (TABLE 3; note the minimal spread between the highest and lowest score). *Cl. feseri* did, however, maintain its superior position, as evidenced by the highest matching score. By combining the data obtained with 2 or more species in many different ways, it was determined that 3 organisms, *Bacteroides pseudoramosus*, *Clostridium feseri*, and *Propionibacterium shermanii* seemed to yield the best possible score while missing the fewest compounds. It is important to note that seven tenths of the materials missed were purine analogues. Thus, these organisms are ill suited for the task of predicting activity for this particular type of compound. The obvious question to follow is: to what type of materials are the anaerobes responsive? We have seen inhibitions caused by steroids, alkylating agents, glucose analogues, and all antitumor antibiotics thus far tested. At present, data regarding these materials are limited; it is hoped that in the near future they can be expanded sufficiently to permit a decision regarding the feasibility of screening with the anaerobes in each of these areas.

In an attempt to elucidate the degree to which anaerobiosis per se may be involved with the activity of some of these compounds, experiments have been conducted using *Escherichia coli B** grown in the agar plate under both aerobic and anaerobic conditions in a glucose-salts medium. The approach is comparable to the use of respiratory-deficient mutants as studied by Gause,[6] the difference being that respiratory energy is denied physically rather than genetically. Tests in this manner with the major antitumor antibiotics have not as yet revealed any pattern of particular sensitivity for the anaerobically grown *E. coli* as compared to that grown aerobically. However, experiments with metabolic inhibitors such as 2-deoxy-D-glucose, 2,4-dinitrophenol, arsenite, azide, and cyanide have provided some interesting observations of an academic nature with both the anaerobes and with binary *E. coli* growth. These findings are being explored further.

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* Supplied by L. Kaplan.

In addition to appraising anaerobe responses as a prediction of antitumor effects, an examination of the data also was conducted with respect to prediction of animal toxicity. The initial finding that inhibition of any 2 of the 3-organism combination correlated with animal toxicity at the 100 mg./kg. multiple dose level with an index of 0.71 suggested that the anaerobes might be somewhat better forecasters of animal toxicity than of antitumor effects. The practical aspects of this hypothesis were studied by reviewing again the data with the 3 organisms on the basis of adjusting the starting dose for the first animal trial in accordance with the degree of bacterial inhibition. Long experience at the institute has established that, for the Sarcoma 180 mouse tumor test, 125 mg./kg./day for 7 days is a useful starting dose when there is no toxicity information available. If no deaths or weight loss occur, the next test is at 500 mg.; or, if toxicity renders the test incomplete, the second test is at one-fourth dose, or 32 mg. From the anaerobe results available with 79 compounds, a starting dose of 32, 125, or 500 was calculated on the basis of the bacterial inhibition noted.

With this scheme, no more than one test could be gained or lost with respect to the usual starting dose. As an illustration, results with several compounds are shown in TABLE 4 as if they had come through as unknown. The table of inhibitory activity against the bacteria used for calculating the doses was selected as the best of several attempts. Compounds giving slight inhibition of 1 organism only, or of none, are entered into animal tests at 500 mg./kg./day. Those inhibiting all 3 organisms maximally (to the "+" or 100 μg. level for this purpose) are run at 32 mg./kg./day. Materials eliciting any of the re-

TABLE 4

PREDICTIVE VALUE OF BACTERIAL INHIBITION FOR ESTABLISHING MTD IN TUMOR-BEARING MICE

Compound	*Bacteriodes pseudoramosus*	*Clostridium feseri*	*Propionibacterium shermanii*	Starting dose mg./kg./day × 7		Observed MTD mg./kg./day × 7	Test gain or loss
				Calculated	Actual		
Nitrogen mustard	+	+	+	32	125	0.75	+1
1,5-Diaminobiuret	+	+	+	32	125	125.00	−1
6-Mercaptopurine	±	−	±	125	125	50.00	
6-Chloropurine	−	−	−	500	125	300.00	+1
DON	+	+	+	32	125	0.05	+1
Diacetylthioguanine	−	−	−	500	125	4.00	−1

<±, ± or +: test at 500 mg./kg./day.
>±, ± or + < +, +, +: test at 125 mg./kg./day.
+, +, +: test at 32 mg./kg./day

sponses in between are tested at the standard 125-mg. dose. Thus nitrogen mustard and 6-diazo-5-oxo-L-norleucine (DON), which gave a + inhibition of all 3 organisms, might have been started at 32 mg. Since the maximum tolerated dose (MTD) of these compounds is considerably below 32 mg., an animal test gain of 1 for each would have been realized. Likewise, the MTD of 6-chloropurine might have been approximated from 2 tests (500 → 250 mg.) instead of 3 (125 → 500 → 250); this exemplifies a test gain from a negative bacterial inhibition result. Obviously there would be test losses (for example, 1,5-diaminobiuret—too low: diacetylthioguanine—too high) and inconsequentials (6-mercaptopurine). The over-all finding with the 79 compounds assessed in this manner was a gain of 20 animal tests versus a loss of 6 for a net gain of 14/79, or 18 per cent, in starting-dose efficiency.

Discussion

Although the inhibitory results obtained with the anaerobes seemed superior to those available from aerobes in a limited comparison,[3] they were not sufficiently striking to permit frank endorsement as a practical screening system. Nevertheless, continued investigation might reveal the areas of sensitivities of a sufficient number of microorganisms to make possible the assembly of a useful combination. As an example, the anaerobes have been generally poor in response to purine antagonists. If they were used together with appropriate *Lactobacillus casei* strains, a screening group might be created thereby that would report a very low percentage of false negatives. The critical value of activity of the system then could be raised to more realistic levels, and matching negatives could be eliminated—the real function of a screening system. Nearly all investigators of nontumor general screening methods have been so preoccupied with matching antitumor activities that they have overlooked this function. Thus, we have given equal meaning to matching negatives in calculating the index used. These were so few in number (vis-à-vis so many false +), however, that a stringent test such as the Youden index[7] in some cases rendered a negative value (per cent true + minus per cent false + < 0), even though the "critical value"[8] of activity used in these experiments gave the best results of many that were tried. It must be pointed out that 76 per cent of the compounds tested were rated as tumor active, whereas only about 10 per cent elicit even a single inhibitory response among all compounds routinely tested *in vivo*. Further investigation with tumor negatives would appropriately balance the picture; or, better still from a practical point of view, an examination of a random collection of unknown materials prior to *in vivo* antitumor tests should be made.

A review of the results suggested that inhibition of the anaerobes might bear some true correlation with respect to animal toxicity. Even if this phenomenon should prove consistent in further studies, there is serious question as to whether the limited gain in animal test efficiency justifies the bacterial screen. However, further exploration of this nature does seem indicated because, again, the group of compounds examined here was heavily weighted with active material. The normal mouse tumor screening operation has a much higher percentage of inactive nontoxic compounds. Thus the possibility exists that the bacterial test might offer much greater improvement in starting-dose efficiency by indicating that most of these materials be sent to *in vivo* trial at a higher dose level.

Summary

Over 100 materials have been tested for inhibition of 10 species of anaerobic bacteria. Results obtained with 90 materials that were also tested against Sarcoma 180 and Adenocarcinoma 755 were made the basis of a comparison between tumor and bacterial inhibition. The over-all correlation was not striking for these bacteria either individually or collectively. *Clostridium feseri* gave the highest matching score of the 10 species tested. A combination of 3 species gave the best available result, a correlation of about 16 per cent better than chance.

A similar study for the prediction of starting dose in animal tests gave an increase in efficiency of 18 per cent with these same 3 organisms as indicators.

Acknowledgments

The compounds used in these studies were received through the courtesy of the following: 6-chloropurine, Cancer Chemotherapy National Service Center, Bethesda, Md.; urethan, Eli Lilly and Company, Indianapolis, Ind.; A-Methopterin, Lederle Laboratories, Pearl River, N. Y.; nitrogen mustard and hydrocortisone, Merck & Company, Inc., Rahway, N. J.; chloramphenicol and DON, Parke, Davis & Company, Detroit, Mich.; *N*-methylformamide and *N*-methylacetamide, Rohm and Haas Company, Philadelphia, Pa.; methylcarbamate and 1,5-diaminobiuret, Southern Research Institute, Birmingham, Ala.; 6-mercaptopurine and diacetylthioguanine, Wellcome Research Laboratories, Tuckahoe, N. Y.

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SPECIAL MICROBIOLOGICAL SYSTEMS. II. OBSERVATIONS ON CHEMICAL MUTAGENESIS IN MICROORGANISMS*

By Waclaw Szybalski

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The chemotherapies of cancer and of infectious diseases are based on a common rationale: destruction, modification, or inhibition of the invader, while the cells of the host are spared. However, the differences between normal and malignant cells are so slight when compared with the great disparity between mammalian and bacterial cells that the search for a sufficiently selective agent becomes a very difficult task. The most significant property of the malignant cell that distinguishes it from the surrounding normal cells is invasiveness, that is, a comparatively high rate of multiplication. Theoretically, this characteristic should make the malignant cell vulnerable to at least two types of agents: those that are selectively toxic and destructive to actively dividing cells, and those that inhibit nonspecifically all cell division and thereby bring the cancer cells into a stationary quiescent state among the normally nondividing surrounding cells. Unfortunately, both these classes of chemotherapeutic agents would inhibit also the normally rapidly regenerating tissues of the host, in particular the hematopoietic system; this type of toxicity is in fact commonly encountered with the majority of the available antineoplastic drugs.

With this bit of theory and great expectations for finding an Ehrlichian "magic bullet" that would inhibit a hypothetical biochemical pathway utilized by the malignant cell, but not by the normal cells of the host, the experimental oncologist generally tends to approach his problem in a rather empirical manner. Although the final test of the efficacy of any antineoplastic agent remains its activity in the diseased human subject, the manner of its preliminary evaluation is not fixed. At present, experimental animal cancers are the initial screening systems of widest usage, but not of greatest facility or economy. Only a few other systems have been evaluated to a comparable extent for preliminary screening of antineoplastic agents. These alternate systems chiefly involve testing for inhibition of microbial growth and cytotoxicity in tissue cultures; they have been discussed elsewhere (Gellhorn and Hirschberg, 1955; Hirschberg, 1955; Reilly, 1953) and are considered in this monograph.

This paper is based principally on two considerations: (1) potential antineoplastic agents may occur with considerably greater frequency among a class of compounds known to possess a specific biological activity than within chemicals chosen at random, and (2) a test for mutagenicity, employing a bacterial mutational system of unusual simplicity, may fill the need for a rapid preliminary screening method. The first contention is supported by the results of a joint evaluation of various biological activities associated with the

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antineoplastic property (Gellhorn and Hirschberg, 1955). Even the class of compounds isolated on the basis of antimicrobial activity contains a proportion of cancerostatic agents that is significantly higher than that found among substances chosen at random (Sugiura *et al.*, 1958).

The rationale for using mutagenic activity as the basis of an anticancer prescreening program is based on several observations. In a survey of twenty-seven compounds, Hemmerly and Demerec (1955) found two potent mutagens, both of which also proved to be quite active in experimental neoplasms; of the six compounds demonstrated to be moderately mutagenic by the Sd-4 mutation test, all but one inhibited some experimental animal cancer. Moreover, antineoplastic agents often have formed the promising pool from which geneticists could select chemical mutagens (Westergaard, 1957). Chemical mutagenicity was first demonstrated as a property of nitrogen mustard (Auerbach and Robson, 1946), one of the earliest antineoplastic drugs. The opposite approach, that is, the use of a test for mutagenicity in a large-scale ancillary screening program for compounds that should be tested against experimental neoplasms, to the best of my knowledge has never been adopted. One reason may have been the lack of highly simplified and rapid methods. The procedure employed here is based on the elegant mutational system used by Demerec *et al.* (1951). Although originally adopted by Iyer and Szybalski (1958a) primarily for the identification of potent mutagens suitable for a study of the kinetics and mechanism of the mutagenic response (Szybalski and Iyer, 1958), the method, by virtue of its simplicity, lends itself to rapid large-scale surveys.

Experimental Methods

Assays for mutagenicity were conducted mainly by the paper-disk method (Iyer and Szybalski, 1958a) or its simplified modification. The increase in the frequency of reversion from streptomycin dependence to independence in strain Sd-4-73 of *Escherichia coli* was utilized as a measure of mutagenicity. Mutagenicity was manifested as a zone of streptomycin-independent mutant colonies around a filter-paper disk saturated with the mutagenic agent and resting on the surface of streptomycin-free nutrient agar seeded heavily with a streptomycin-dependent parental population. The inoculum consisted of an overnight culture of strain Sd-4-73 grown at 36° C. in aerated nutrient broth containing 20 μg./ml. of streptomycin. The culture was centrifuged and washed at least twice with saline or distilled water to remove the extraneous streptomycin, and was resuspended in saline to a concentration of approximately 10^9 cells/ml. A 0.1-ml. aliquot of this suspension was mixed with 2.5 ml. of molten soft nutrient agar (0.7 per cent agar) and poured over a base of 20 ml. of 1.5 per cent nutrient agar. Additional plates were prepared with small inocula (one fifth and one twenty-fifth of the original) so as not to miss the optimum cell density; the number of cells per plate was rather critical, the yield of mutant colonies being reduced either by crowding or by insufficient population size. After the soft agar layer had set, the mutagen, in the form of a microdrop of solution (0.01 to 0.025 ml.) or a small crystal, was applied to a small filter-paper disk resting on the agar, as previously described by Iyer

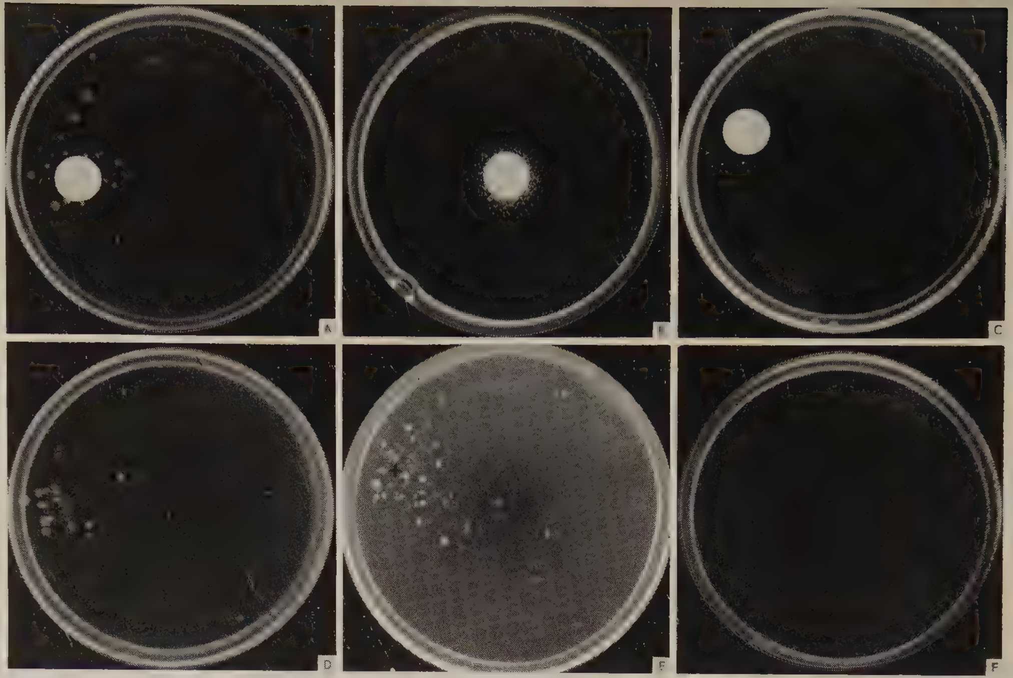

FIGURE 1. Direct test for mutagenicity with (A to C) or without (D to F) use of the paper disk. All plates contain 20 ml. of nutrient agar (1.5 per cent agar) with a 2-ml. overlay of soft agar (0.6 per cent agar) seeded with approximately 10^7 cells of streptomycin-dependent strain Sd-4 of *E. coli*. Plates C and F represent control experiments for both series (A to C and D to F) with no mutagen added, and only a few or no revertant colonies showing. Plates A and B demonstrate the appearance of a zone of colonies of streptomycin-independent revertants when 30 μg. of sulfocidin (A) or a concentrated filtrate of an unidentified fungus (B) was added to the disk. Plates D and E represent the effect of 100 μg. of TEM and ethylenimine, respectively. The hazy circular area at the spot of application of TEM to the agar (D) apparently is due to a chemical interaction between some constituent of nutrient agar and TEM. These prints were obtained by placing Petri dishes directly in a photographic enlarger.

TABLE 1

COMPARISON OF PAPER-DISK TEST WITH MUTAGENICITY ASSAY*

	Mutagenic effect	
	Paper-disk test†	Method of Demerec *et al.*‡
Actinomycin	−	−
Aminopterin	−	−
A-Methopterin	±	±
Ammonium hydroxide	±	−
Alazopeptin	−	−
8-Azaguanine	−	−
Azaserine	+++	+++
6-Azathymine	−	−
6-Azauracil	−	−
Benzimidazole	±	−
Boric acid	−	−
Caffeine	−	−
Carzinophilin	+	++
6-Chloropurine	±	+
Cupric sulfate	−	−
2,4-Diamino-5-*p*-chlorophenyl-6-ethylpyrimidine	−	−
2,4-Diamino-5-(3,'4'-dichlorophenyl)-6-ethylpyrimidine	±	±
2,6-Diaminopurine	−	±
6-Diazo-5-oxo-L-norleucine	−	−
2,5-Bis-ethyleniminobenzoquinone	++	++
Formaldehyde	±	++
D-Glucosamine	−	−
Hydrogen peroxide	±	+
Kinetin	−	−
6-Mercaptopurine	−	−
N-Methyl-di-2-chloroethylamine·HCl	+++	+++
N-Methyl-di-2-chloroethylamine-*N*-oxide·HCl	++	++
Myleran	±	±
Nucleocidin	−	−
Proflavine (sulfate)	±	−
Beta propiolactone	+++	++
Puromycin	−	−
Sulfocidin	++	++
Triethylenemelamine	+++	+++
Trypaflavine	−	−
Urethan	−	±

* The method of mutagenicity assay is that of Demerec *et al.* (1951), with minor modifications described by Iyer and Szybalski (1958a). The comparison is based on the results of Iyer and Szybalski (1958a) and several subsequent experiments.

† The symbol − indicates lack of mutagenic effect (relative mutagenicity equal to 1). The signs ±, +, ++, and +++ correspond to relative mutagenicities of 1 to 2, 2 to 4, 4 to 10, and over 10, respectively. Relative mutagenicity is defined as an approximate ratio of the number of colonies on the plate containing the mutagen to the number of colonies on the control plate. When induced mutant colonies form a definite ring around the paper disk, the figure for relative mutagenicity is multiplied by the ratio of the total area of the plate to the area of the growth of induced mutant colonies.

‡ The signs ±, +, ++, and +++ correspond to frequencies of induced mutants of less than 10, 10 to 50, 50 to 500, and over 500, respectively, per 10^8 treated surviving cells. The minus sign indicates no increase in the number of mutants over the untreated controls.

and Szybalski (1958a), or was placed directly on the agar in the simplified modification of the method. To determine whether the substance was inhibitory for the assay organism at the concentration employed, the procedure was repeated on a nutrient agar plate containing 100 μg./ml. of streptomycin and seeded with approximately 10^7 bacteria. FIGURE 1 shows the results obtained.

The paper-disk method was compared with the quantitative method of Demerec *et al.* (1951), using a selected group of 36 agents. Satisfactory correlation between these 2 methods was demonstrated by Iyer and Szybalski (1958a) as summarized in TABLE 1.

Correlation Between the Mutagenic and the Antineoplastic Properties

Up to the time of the preparation of this paper, 431 substances had been tested for mutagenicity by the paper-disk method. These substances, listed in TABLE 2, were selected from compounds available in my laboratory, and included large numbers of antibiotics, purine and pyrimidine analogues, ethylenimino derivatives, nitrogen mustards, amino acids and their analogues, benzimidazoles, nitrofurans, and many other natural and synthetic products. The sources of most of the compounds are indicated in the *Acknowledgments*; the more common chemicals were obtained through regular commercial channels.

Twenty-two substances showed pronounced mutagenicity when assayed on the Sd-4 and Sd-4-73 strains of *E. coli*, as evidenced by the appearance of mutant colonies around the site of application of the compound. The relative mutagenicity (in arbitrary units as defined in TABLE 1) of these potent mutagens was greater than 3. The 40 substances classified as weak and doubtful mutagens displayed relative mutagenicities between 1.5 and 3; the zone of mutant colonies was not clearly visible in all experiments. Thus, only 5.1 per cent of the 431 substances tested were definitely mutagenic under the conditions of the experiments; 9.3 per cent showed weak or doubtful mutagenicity; and 85.6 per cent displayed no activity. These results are summarized in TABLE 3.

More interesting was the distribution of the "known antineoplastic agents" among these 3 classes. The designation "known antineoplastic agent" was based on data either reported in the literature (particularly the review of Farber *et al.*, 1956) or supplied by the donor of the compound (see *Acknowledgments*), but it requires some qualification. Not all the substances surveyed here have been tested in experimental cancer systems; moreover, the results of such tests are highly dependent on the particular tumor strain and host employed. Of the total compounds tested, only a few have been reported to be beneficial in human neoplastic disease. Of 22 compounds that proved to be definitely mutagenic in our experiments, 19 (86.4 per cent) belonged to the group of known antineoplastic agents. This was a striking accumulation of compounds with antineoplastic activity in a single class, when contrasted with the much lower incidence of such agents in the entire group and in the class of nonmutagens (14.4 per cent and 6.8 per cent, respectively). The other 3 potent mutagens (β-propiolactone, ethylenimine, diepoxybutane) either have not been tested in experimental cancer systems or have shown negative or borderline activity. The class of weak and doubtful mutagens also was heavily endowed with antineoplastic compounds (45 per cent).

If, as a follow-up of the present study, only the pronounced mutagens were selected for evaluation as antineoplastic agents, it would be necessary to test 22 compounds (5.1 per cent of the total) in experimental cancer systems, and the yield of known antineoplastic agents (19) would comprise 30.7 per cent of all such agents in the entire group. An evaluation of both the strong and weak mutagens (62 compounds) would result in recovery of 59.7 per cent of

TABLE 2

SUBSTANCES TESTED FOR MUTAGENICITY AND CLASSIFIED AS MUTAGENS, WEAK OR DOUBTFUL MUTAGENS, AND NONMUTAGENS

Antibiotics and Antibioticlike Substances

Mutagens
- Azaserine
- Mitomycin
- Sulfocidin

Weak or doubtful mutagens
- Amicetin
- Carzinophilin
- Chartreusin
- 4-Nitroquinoline-1-oxide
- 4-Nitroquinaldine-1-oxide
- Sarkomycin
- Thiolutin

Nonmutagens
- Acti-dione
- Actinomycin D
- Alazopeptin
- Amphomycin
- Anhydrotetracycline
- Antibiotic NA-255-B8
- Antibiotic AG-35B
- Antibiotic PA-89
- Antibiotic NA-1075-X34
- Antibiotic X-206
- Antibiotic X-464
- Antibiotic X-537-A
- Bacitracin
- Carbomycin
- Catenulin
- Chloramphenicol
- Chlortetracycline
- Cinnamycin
- Cycloserine
- 6-Diazo-5-oxo-L-norleucine (DON)
- Erythromycin
- Fradicin
- Fumidil

Nonmutagens—Continued
- Gramicidin
- Grisein
- Illudin M
- Illudin S
- Kanamycin
- Licheniformin B
- LPF-factor
- Marasmic acid
- 5-Methoxytoluquinone
- Micrococcin
- Mycomycetin
- Mycolutein
- Neamine (neomycin A)
- Neomycin B
- Neomycin C
- Netropsin
- Novobiocin
- Nucleocidin
- Oxytetracycline
- Patulin
- Pleocidin
- Pleuromutilin
- Podophyllotoxin-β-*d*-glucoside
- Polymyxin B
- Polymyxin E
- Puromycin (Stylomycin)
- Pyrexin
- Streptolydigin
- Streptothricin
- Streptovaracin
- Subtilin
- Tetracycline
- Thioaurin
- Tyrocidine
- Viomycin
- Xanthomycin

Purines

Mutagens
- 6-Mercaptopurine riboside

Weak or doubtful mutagens
- 7-Methylhypoxanthine (W-66)
- 6-Ethylmercapto-7-methyl purine (W-102)
- 7-Methyl-6-methylaminopurine (W-141)
- 6-Chloro-7-methyl purine (W-103)
- Theophylline

Nonmutagens
- 6-Azathymine
- 8-Azaguanine

Nonmutagens—Continued
- Caffeine
- 6-Chloropurine
- 2,6-Diaminopurine
- 2:6-Dichloro-7-methyl purine (W-19A)
- 6-Furfurylaminopurine (kinetin)
- 7-Methyladenine (W-132)
- 6-Mercaptopurine (6-MP)
- 7-Methyl-2:6-diaminopurine (W-80)
- 7-Methyl-6-mercaptopurine (W-126)
- 7-Methyl-2-thiohypoxanthine (W-147)

Pyrimidines

Weak or doubtful mutagens
- 4-Butylaminopyrazolo-3:4-d-pyrimidine (O-14)
- 2,4-Diamino-5-(3′,4′-dichlorophenyl)-6-ethylpyrimidine

Weak or doubtful mutagens—Continued
- 5-Fluorouracil
- 5-Hydroxyazouracil
- 4-Methylamino-1-methylpyrazolo-3:4-d-pyrimidine (A-106)

TABLE 2—*Continued*

Pyrimidines—*Continued*

Nonmutagens
- 4-Aminopyrazolo-3:4-d-pyrimidine (A-31)
- 4-Amino-1-methylpyrazolo-3:4-d-pyrimidine (A-74)
- 6-Amino-2,4-pyrimidinediol
- 6-Aminothiouracil
- 5-Aminouracil
- 6-Azauracil
- 5-Chlorouracil
- 6-Cyclopropyl-2-thiouracil
- 5,6-Diamino-2-thiouracil
- 2,4-Diamino-6-(*p*-chloroanilino)pyrimidine
- 2,4-Diamino-5-*p*-chlorophenyl-6-ethylpyrimidine (Daraprim)
- 5-Fluoroorotic acid
- 6-(Methylsulfonyl)-2,4-pyrimidinediol

Nonmutagens—Continued
- 6-Methyl-2-thiouracil
- 6-Methyluracil
- 5-Nitrouracil
- 6-Propyl-2-thiouracil
- 2-Thiothymine
- 2-Thiouracil
- Bis-1,3-β-ethylhexyl-5-methyl-5-aminohexahydropyrimidine (L-6)
- Bis-1,3-*n*-octyl-5-methyl-5-aminohexahydropyrimidine (L-499)
- Bis-1,3-(1-methylpropyl)-5-methyl-5-myristamidohexahydropyrimidine (L-505)
- 1,3-Bis-(1-methylheptyl)-5-methyl-5-aminohexahydropyrimidine (L-509)
- Bis-1,3-*n*-heptyl-5-methyl-5-aminohexahydropyrimidine (L-523)

Nitrogen Mustards

Mutagens
- Di-2-chloroethylamine·HCl (CB 1153)
- *N*-Methyl-di-2-chloroethylamine·HCl (HN2)
- *N*,*N*-Di-(2-chloroethyl)-*p*-aminophenylethyldimethylamine·HCl (CB 3039/1)
- *N*,*N*-Di-(2-chloromethyl)-*p*-aminophenylethylamine·HCl (CB 3034)
- *N*-Methyl-di-2-chloroethylamine-*N*-oxide·HCl (Mitomen, Nitromin)

Weak or doubtful mutagens
- d-*N*,*N*-Di-(2-chloroethyl)-*p*-aminophenylalanine(Merphalan) (CB 3026)
- *N*,*N*-Di-(2-chloroethyl)-*p*-aminophenylethyl-*N*′-monomethylamine (CB 3050)
- *N*,*N*-Di-2-chloroethyl-*p*-aminophenylbutyric acid (Chlorambucil) (CB 1348)
- 5-Chloro-1-(chloromethyl)-*n*-hexyl-2-chloroethylamine·HCl
- 5-Chloro-1-(chloromethyl)-*n*-pentyl-2-chloroethylamine·HCl

Nonmutagens
- 1(*N*,*N*-Di-2′-chloroethylamino)-4-trichloroacetylaminobenzene (CB 1431)
- 1-*N*,*N*-Di-(2-chloroethyl)-*p*-aminophenylalanine (Melphalan) (CB 3025)
- *N*,*N*-Di-2-chloroethyl-*p*-toluidine (CB 1044)
- *N*,*N*-Di-2-chloroethyl-*o*-toluidine (CB 1051)
- *N*,*N*-Di-2-chloroethyl-*p*-aminobenzoic acid (CB 1078)

Nonmutagens—Continued
- *N*,*N*-Di-2-chloroethyl-*p*-aminophenylacetic acid (CB 1331)
- Ethyl-*N*,*N*-di-2-chloroethyl-*p*-aminophenylacetate (CB 1330)
- γ-(*p*-*N*,*N*-Di-2-chloroethylaminophenyl)-α-aminobutyric acid (CB 1385)
- *p*-*N*,*N*-Di-2-chloroethylaminobenzaldoxime (CB 1454)
- *N*-Fluoroacetyl-*N*′,*N*′-di-2-chloroethyl-*p*-phenylenediamine (CB 1497)
- *N*,*N*-Di-2-chloroethyl-*p*-aminoazobenzene (CB 1124)
- 4-(*N*,*N*-Di-2″-chloroethylamino)-2-methylazobenzene (CB 1413)
- 4-(*N*,*N*-Di-2″-chloroethylamino)-2-methyl-2′-carboxyazobenzene (CB 1414)
- 4-(α-Naphthylazo)-3-methyl-*N*,*N*-di-2″-chloroethylamine (CB 1444)
- 4-(*N*,*N*-Di-2″-chloroethylamine)-3-methoxy-3′-carboxyazobenzene (CB 1500)
- 4-(*N*,*N*-Di-2″-chlorethylamino)-3-methoxy-3′-nitroazobenzene (CB 1505)
- 1-(*N*,*N*-Di-2′-chloroethylamino)-3-methyl-4-phenylureidobenzene (CB 1471)
- β-Naphthyl-di-2-chloroethylamine (CB 1048)
- β-Naphthyl-di-2-bromoethylamine (CB 1067)
- β-Naphthyl-di-2-chloroisopropylamine (CB 1075)

Ethylenimine Derivatives

Mutagens
- Ethylenimine
- 1:6-Hexamethylene-di(*N*,*N*′-ethyleneurea) (Hoechst 105, CB 1263)
- Triethylenephosphoramide (TEPA)

Mutagens—Continued
- Triethylenethiophosphoramide (TSPA, Thio-TEPA)
- 3-Oxapentamethylene-*N*′,*N*″-diethylenethiophosphoramide (MSPA)

TABLE 2—*Continued*

Ethylenimine Derivatives—*Continued*

Mutagens—Continued
- 3-Oxapentamethylene-*N′*,*N″*-diethylenephosphoramide (MEPA)
- *N*,*N′*-Diethylene-*N″*-phenylphosphoramide (DPPA)
- Triethylenemelamine (2,4,6-triethylenimino-*s*-triazine) (Hoechst 193, TEM)

Mutagens—Continued
- 2,5-Bis-ethyleniminobenzoquinone

Nonmutagens
- 2,5-Bis-*n*-propoxy-3,6-bis-ethyleniminobenzoquinone (E-39)
- 2,5-Bis-methoxyethoxy-3,6-bis-ethyleniminobenzoquinone (A-139)
- *n*-Nonadecylethylenimide (Hoechst 185)

Miscellaneous Chemicals

Mutagens
- 1,2-3,4-Diepoxybutane
- 6-Hydroxy-4-nitrobenzimidazole
- 1-Phenyl-3,3-dimethyltriazene (SK-11687d)
- β-Propiolactone

Weak or doubtful mutagens
- A-Methopterin (Methotrexate)
- 2-Aminofluorene (AF)
- 2-Succinamidofluorene (SAF)
- Ammonium hydroxide
- Benzimidazole
- *N*-Cocomorpholine
- α,β-Dichloro-γ-(3,4-dimethoxyphenyl)-$\Delta^{\alpha\beta}$-crotonolactone
- α,β-Dichloro-γ-*p*-methoxyphenyl-$\Delta^{\alpha\beta}$crotonolactone
- 1,4-Dimethanesulfonoxybutane (Myleran, CB 2041)
- Ethomeen 0/15
- Ethomeen 0/25
- Formaldehyde
- Hydrogen peroxide
- 5-Hydroxy-4-nitrobenzamidazole
- 4-Hydroxy-6-nitrobenzamidazole
- 5-Nitro-2-furaldehyde-4-(2-hydroxyethyl)semicarbazone (NF-145)
- *N*-(5-Nitro-2-furfurylidene)-3-amino-2-oxazolidone (NF-180)
- Proflavine sulfate
- α-Sulfopalmitic acid

Nonmutagens
- Allylamine
- Aldinamide
- Alizarin yellow
- Aluminum ethoxide
- *p*-Aminoacetophenone
- 1-Aminoanthraquinone
- 4-Aminoantipyrine
- 2-Amino-5-azoanisole
- *p*-Aminoazobenzene
- 2-Amino-6-hydroxycaproic acid
- Aminooxyacetic acid hemihydrochloride
- *p*-Aminophenylmercuric acetate
- 3-Aminophthalhydrazide
- Aminopterin
- 2-Aminopyridine
- 3-Aminoquinoline
- 4-Aminosalicylic acid

Nonmutagens—Continued
- *n*-Amyl alcohol
- Isoamyl benzoate
- *n*-Amyl bromide
- α-*n*-Amyl cinnamaldehyde
- 2-Anthraquinonecarboxylic acid
- Armeen DMCD
- Armeen DM16D
- 7-Azaindol
- Azelaic acid
- Azobenzene
- Azoxybenzene
- Barbituric acid
- Barium diphenylaminosulfobenzoate
- Benzeneazoresorcinol
- Benzenephosphonous acid
- Benzenesulfinic acid
- Benzenesulfonamide
- Benzenesulfonyl chloride
- Benzenethiol
- 1,2,4-Benzenetriamine dihydrochloride
- Benzhydrol
- Benzyl
- Benzylic acid
- α-Benzylmonoxime
- Bis(2,4-dinitrophenyl) disulfide
- Bis(2-ethylbutyl)phthalate
- Bis(2-methoxyethyl) carbonate
- Boric acid
- Boronfluoroethyl ether
- Bromoacetic acid
- 1-(*p*-Butoxyphenyl)-3-(*p*-dimethylaminophenyl)-2-thiourea
- *n*-Butyl sulfone
- *n*-Butylvinyl ether
- *n*-Butyraldehyde
- Isobutyraldehyde
- *n*-Butyraldoxime
- *n*-Butyramide
- *n*-Butyric acid
- Isobutyric acid
- Captan
- Carbanilide
- Carbazole
- Carbethoxymethyl isocyanate
- 6-Chlorothymol
- Cholesterol
- Choline chloride
- Chrysazin

TABLE 2—*Continued*

Miscellaneous Chemicals—*Continued*

Nonmutagens—Continued

- Colchicine
- Congo red
- Coumarilic acid
- α,β-Dichloro-γ-*p*-hydroxyphenyl-$\Delta^{\alpha\beta}$-crotonolactone
- α,β-Dichloro-γγ-di(*p*-chlorophenyl)crotonic acid
- Crotonic acid
- Crystal violet
- Cupferron
- Cyclohexanebutyric acid
- Cupric sulphate
- *p*-Cymeme
- Decanoic acid
- *n*-Decyl alcohol
- Dehydroacetic acid
- Deoxybenzoin
- 2,4-Dibromophenol
- 2,6-Dibromoquinone chlorimide
- Di-*n*-butylamine
- *N*,*N*-Di-*n*-butylacetamide
- Di-*n*-butyl carbonate
- 2,6-Di-(tert.)-butyl-*p*-cresol
- Diethylenetriamine
- Diethylmalonate
- Diethylmethyloxalacetate
- Diethyloxalacetate
- *N*,*N*-Diethyl-*p*-phenylenediamine
- Diethylphthalate
- 2,2-Diethyl-1,3-propanediol
- Diethylsebacate
- Diethylsuccinate
- Diethylsulfate
- Dimethanesulfonamide
- 2,4-Dimethoxybenzoic acid
- 2,6-Dimethoxyphenol
- *N*,*N*-Dimethylacetamide
- Dimethylamine
- *p*-Dimethylaminobenzaldehyde
- 2-Dimethylaminoethanol
- 1,4-Dimethyl-1,4-dimethanesulfonoxybutane (dimethyl Myleran) (CB 2348)
- *N*,*N*-Dimethyl-*m*-nitroaniline
- *N*,*N*-Dimethyloctadecylamine
- Dimethyloxalate
- 2,4-Dimethylphenol
- *N*,*N*-Dimethyl-*p*-phenylazoaniline
- 1,2-Dithiolane-5-methyl-3-carboxylic acid
- 1,2-Dithiolane-3-carboxylic acid
- Ephedrine
- *p*-Ethoxyacetophenone
- *o*-Ethoxybenzaldehyde
- 2-Ethoxyethanol
- Ethomeen C/25
- Ethomeen O/12
- *p*-Ethylacetophenone
- Ethylamine
- *N*-Ethylaniline

Nonmutagens—Continued

- *p*-Formylacetanilide-3-thiosemicarbazone
- Fumaric acid
- Gantrisin
- d-Glucosamine
- Guanidine acetate
- 2-Hydroxymethylphenylboric acid
- 4-Hydroxy-3-nitrobenzenearsonic acid
- *p*-Hydroxyphenoxyacetic acid
- 2,2′-(*p*-Hydroxyphenylimino)diethanol
- *p*-Hydroxypropiophenone
- Isonicotinic acid hydrazide (Isoniazid)
- 1-(*p*-Isobutoxyphenyl)-3-(*p*-phenethyl)-2-thiourea
- 2,4-Lutidine
- 2,6-Lutidine
- Malachite green oxalate
- Maleic acid
- Maleic acid hydrazide
- Mandelamine
- Mercaptoacetic acid
- *o*-Mercaptobenzoic acid
- 2-Mercaptobenzothiazole
- Mercaptosuccinic acid
- Metanil yellow
- Methanesulfonic acid
- 3-Methoxypropylamine
- 8-Methoxypsoralen (8-MOP)
- 2-*N*-Methylanilinoethanol
- Methyl anisate
- *N*-Methylanthranilic acid
- 2-Methylanthraquinone
- Methyl benzoate
- DL-α-Methylbenzyl alcohol
- Nicotinaldehydethiosemicarbazone
- Nicotinic acid
- Ninhydrin
- 5-Nitroacenaphthene
- 5-Nitro-2-furaldehydesemicarbazone (Furacin, NF-7)
- 5-Nitro-2-furaldehyde-2-(2-hydroxyethyl)semicarbazone (Furadroxyl, NF-67)
- 5-Nitro-2-furaldehydetrimethylammonium chloride (NF-114)
- *N*-(5-nitro-2-furfurylidene)-1-aminohydantoin (NF-153)
- 5-Nitro-2-furaldehyde-4-(3-diethylaminopropyl)semicarbazone·HCl (NF-185)
- *o*-Nitrophenol
- *m*-Nitrophenol
- *p*-Nitrophenol
- 6-Nitroquinoxaline
- 3-Nitrosalicylic acid
- *N*-Nitrosodiphenylamine
- Phenylhydrazine hydrochloride
- Phenylhydroquinone
- Phenylisocyanate
- Phenylmercaptoacetic acid

TABLE 2—*Continued*

Miscellaneous Chemicals—*Continued*

Nonmutagens—Continued

Phenylpropionate
6-Phenylquinoline
Phenylsalicylate
Pimelic acid
Piperazine
Piridylmercuric acetate
Prednisone
Prednisolone
6-Methylprednisolone (Medrol)
Propionic acid
Propionitrile
Quinalizarin
Quinoline
Quinoxaline
Resazurin
β-Resorcylic hydrazide
Riboflavin
Rosaniline chloride
Rutin
Skatole
Sodium allylsulfonate
Sodium azide
Sodium bisulfite
Sodium dicyanamide
α-Sulfostearic acid
Tetrabromo-*o*-cresol
2,4,7-Trinitro-9-fluorenone
2,4,6-Trinitroresorcinol
s-Trioxane
Triphenylamine
Triphenyl phosphate
Triphenyl phosphite
2,3,5-Triphenyl-2*H*-tetrazolium chloride
Triphenyltin chloride
Trypaflavine
Urea
Urethan
Vanillin
Veratrole
Violuric acid
Acetylglycine

Nonmutagens—Continued

N-Acetyl-N-phenylglycine
DL-α-Alanine
β-Alanine
DL-α-Alanine ethyl ester hydrochloride
DL-α-Amino-*n*-butyric acid
α-Aminoisobutyric acid
DL-2-Aminohexanoic acid
p-Aminohippuric acid
DL-2-Aminooctanoic acid
DL-α-Aminophenylacetic acid
DL-α-Amino-*n*-valeric acid
L-(+)-Arginine monohydrochloride
DL-Asparagine
DL-Aspartic acid
7-Azatryptophan
DL-N-Benzoyl-α-alanine
Betaine hydrochloride
Creatine
Creatinine
Cysteine hydrochloride
L-(−)-Cystine
DL-β-(3,4-Dihydroxyphenyl)alanine
L-(+)-Glutamic acid hydrochloride
Glutathione
Glycine
Hippuric acid
L-(+)-Histidine monohydrochloride
N-(*p*-Hydroxyphenyl)glycine
DL-Leucine
DL-iso-Leucine
L-(+)-Lysine dihydrochloride
DL-β-Phenylalanine
N-Phenylglycine
2,5-Piperazinedione
DL-Serine
Taurine
DL-Threonine
DL-Tryptazan
DL-Tryptophan
DL-Tryosine
DL-Valine

all the known antineoplastic agents from among the 431 compounds. Obviously, these figures are representative only of a particular group of substances, but they point unquestionably to a correlation between antineoplastic activity and mutagenicity.

A few of the important antineoplastic agents, such as 6-mercaptopurine (6-MP) and 6-diazo-5-oxo-L-norleucine (DON), were not found among the mutagens. Cellular permeability might be involved in the case of 6-MP, especially since 6-MP-riboside showed definite mutagenicity. DON is a highly unstable substance; under specially designed experimental conditions, a mutagenic effect might have been demonstrated. The problem of instability can be illustrated by reference to the antibiotic azaserine that was pro-

TABLE 3

INCIDENCE OF KNOWN ANTINEOPLASTIC AGENTS WITHIN CLASSES OF MUTAGENIC AND NONMUTAGENIC SUBSTANCES

		Classes			Total
		Mutagens		Non-mutagens	
		strong	weak		
	Number of substances	22	40	369	431
	Percentage of total	5.1 %	9.3 %	85.6 %	100 %
Antineoplastic agents	Number in class	19	18	25	62
	Percentage of class	86.4 %	45.0 %	6.8 %	14.4 %
	Percentage of total number of antineoplastic agents	30.7 %	29.0 %		
		59.7 %			

gressively inactivated under the present experimental conditions (Iyer and Szybalski, 1958b); its mutagenicity, although pronounced, might have been missed had the rate of decomposition been more rapid.

It was previously reported that actinomycin D is a potent antimutagen, while azaserine acts synergistically with manganous chloride (Steinman *et al.*, 1958). The behavior of actinomycin D, which at the 1-μg./ml. level counteracts the mutagenic effect of 400 μg./ml. of manganous chloride, suggests that the study of antimutagenicity also may point to a possible correlation between that property and antineoplastic activity. The present assay system also was applied to the analysis of interactions among mutagens, and between mutagens and nonmutagens; for this purpose, two compounds were applied to the same test plate in such a manner that their zones of activity somewhat overlapped. In a limited number of tests, no pronounced antagonistic or synergistic interaction was observed between various paired combinations of triethylenemelamine (TEM), nitrogen mustards, ethylenimine, theophylline, azaserine, 4-nitroquinoline-1-oxide, and 5-hydroxyazauracil. There were indications of slight synergistic mutagenic interactions between TEM and 6-chloropurine, TEM and 6-hydroxy-4-nitrobenzimidazole, and TEM and 1-phenyl-3,3-dimethyltriazene (SK 11687d).

Conclusions

The results presented here point to the usefulness of the described test for mutagenicity as a simple preliminary screen in the search for prospective antineoplastic agents. The method is adapted to the survey of pure or complex materials available in abundant numbers, such as fermentation liquors, natural products, and shelf organic chemicals listed in commercial catalogues. The incidental observation that a colony of a particular mold contaminant induced the appearance of a ring of revertant colonies of the Sd-4-73 strain suggests the possibility of a more direct screening procedure for mutagens from microbial sources; large numbers of microorganisms could be so tested without the necessity of preparing sterile filtrates.

The simplicity of the present assay system is emphasized by the fact that a single laboratory assistant can easily test fifty compounds per day with minimal use of materials. Quantitation of the paper-disk method might facilitate the recovery and purification of the mutagenic factor contained in a natural substrate, particularly when other methods are not applicable.

The same basic principles could be applied to develop a test for antimutagens and promutagens, that is, substances that will counteract or boost, respectively, the mutagenicity of another compound (Steinman *et al.*, 1958); in this manner, other groups of compounds with interesting biological activity and possible usefulness in the field of cancer chemotherapy would be selected.

The striking difference in activity between 6-MP and its riboside derivative points to the necessity of evaluating, not only the purine and pyrimidine analogues, but also their more sophisticated nucleosidelike and nucleotidelike complexes. Microbiological synthesis of the latter, a comparatively easy task, is at present the most promising approach to the rational improvement of these important antineoplastic agents. The difference in antimutagenic activity between purines and their ribosides also was noted by Novick (1955).

The examination of two rather large groups of compounds, the nitrogen mustards and the ethylenimino derivatives, permits the drawing of some conclusions regarding the possible association of mutagenicity with particular chemical groupings. The great majority of the ethylenimino derivatives proved to be potent mutagens, but only a small proportion of the nitrogen mustards were mutagenic. The effect of substitutions and ionic states on the solubility of nitrogen mustards and their mutagenic effect have been studied insufficiently at this time.

The pronounced activity of the ethylenimino grouping suggests the desirability of testing its effect following incorporation into substances reported to be specifically accumulated by tumors, such as the tetracyclines (Rall *et al.*, 1957), assuming that the activity of the carrier compound would be preserved.

It must be borne in mind that the mutagenic and antineoplastic activities have one more property in common: dependence on the biological system. Drugs effective against one type of neoplasm are ineffective against another. Similarly, some "mutons" respond well to one type of mutagen, while others are refractory to the same agent, but susceptible to another (Demerec, 1954). These findings point to the desirability of using more than one mutational system. Other systems that would be suitable for the survey of complex natural products containing nutrient materials include mutation to bacteriophage resistance or drug resistance, and reversions from nutritional deficiencies for very rare substrates. If the simplicity of the assay could be preserved, mutational systems employing different groups of organisms might be employed. At present I am studying the adaptability of human cell lines for a simple mutagenicity assay, since obviously a closer correlation could be expected between activity in the human organism and the results of *in vitro* tests on human cell lines.

The variety of responses exhibited by different tumor types magnifies the difficulty in finding a "magic bullet" against neoplastic disease. Cancer chemotherapy is perhaps an engineering problem in which specific drugs or, rather, rational combinations of drugs must cope with different types of tumors and secondary problems, such as the development of drug-resistant cells.

Summary

A simple method for the assay of mutagenicity permits rapid testing of a large number of chemical substances. The method employs streptomycin-dependent mutants of *Escherichia coli*, which cannot grow on streptomycin-free nutrient agar, but will produce a zone or a ring of streptomycin-independent revertant colonies around a drop or crystal of mutagen on the agar. This simple assay permits evaluation of both pure compounds and complex natural products.

In a survey of 431 substances, 22 (5.1 per cent) were found to be effective mutagens, 40 (9.3 per cent) were designated as weak or doubtful mutagens, and 369 (85.6 per cent) did not display any mutagenic activity under the conditions of the assay system. The distribution of the known antineoplastic agents between the mutagen and nonmutagen classes suggested a striking correlation between the mutagenic and antineoplastic properties. Known antineoplastic agents comprised 86.4 per cent of the strong mutagen class, 45.0 per cent of the weak and doubtful mutagen class, but only 6.8 per cent of the nonmutagen class. Thus, by means of the mutagenicity assay it is possible to select a small group of substances, a considerable proportion of which can be demonstrated by appropriate tests to possess antineoplastic activity.

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SPECIAL MICROBIOLOGICAL SYSTEMS. III. THE USE OF LACTIC ACID BACTERIA FOR THE EVALUATION OF PURINE AND PYRIMIDINE ANTAGONISTS

By George H. Hitchings
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The use of *Lactobacillus casei* to evaluate potential antimetabolites related to the purine and pyrimidine bases and folic acid has been described in earlier publications.[1-9] *L. casei* requires folic acid for its nutrition. However, this requirement can be replaced by the substitution of a purine and the pyrimidine, thymine. The organism is capable, therefore, of synthesizing its purine and pyrimidine requirements *de novo*, or of incorporating preformed purine and pyrimidine moieties.[10-14] As a result, it has been possible to use this organism to detect and characterize a variety of antimetabolic activities related to nucleic acid biosynthesis, using only a few milligrams of material and some minor variants of a single basal medium.

In FIGURE 1 the effects of various additions to three of the control media are shown. Growth is measured as acid production and represented as the titer of 10 ml. of culture and is shown along the horizontal axis. The maximum value of about 14 ml. in this parameter represents essentially the stoichiometric conversion of the available glucose to lactic acid. None of these control media supports growth in itself; they are used chiefly to detect the presence of metabolites in the supplementary substances being tested. The presence of folic acid is detected by growth that is essentially equal in all three media. Parenthetically, this has proved itself to be a very sensitive test for contamination with folic acid, as little as 5 parts in 10^8 being readily apparent—a concentration that occurs in many substances isolated from natural sources and has been found in synthetic chemicals from several laboratories. The addition of adenine or its equivalent is detected by growth in the OT medium, and the presence of thymine is detected by growth in the P medium. In fact, the addition of both adenine and thymine in the amounts indicated in FIGURE 1 produces the PT medium, which is useful in the characterization of antimetabolites.

The use of four additional media designed primarily to detect antimetabolite effects is illustrated in FIGURE 2. Here, growth is represented on the horizontal axis, and the control growth is shown on the bottom line. It will be observed that, in OFA medium, growth is about 6 or 7 ml., that is, about half maximal. FA^+ contains sufficient folic acid (0.625 mγ/ml.) to produce maximal growth. PFA is the OFA solution with the addition of purine (adenine, 6.7 γ/ml.), which produces only slightly greater growth than the OFA medium. PT is the thymine-purine medium, which has been described. It can be seen that 5-bromouracil (top line) is identified as a thymine antagonist primarily because of the inhibition of growth in the PT medium. 6-Azauracil inhibits growth in all, and another type of experiment is required to demonstrate the nature of the inhibition. This can be done easliy when it is shown that the inhibition is reversed competitively by uracil. Diaminopurine inhibits the

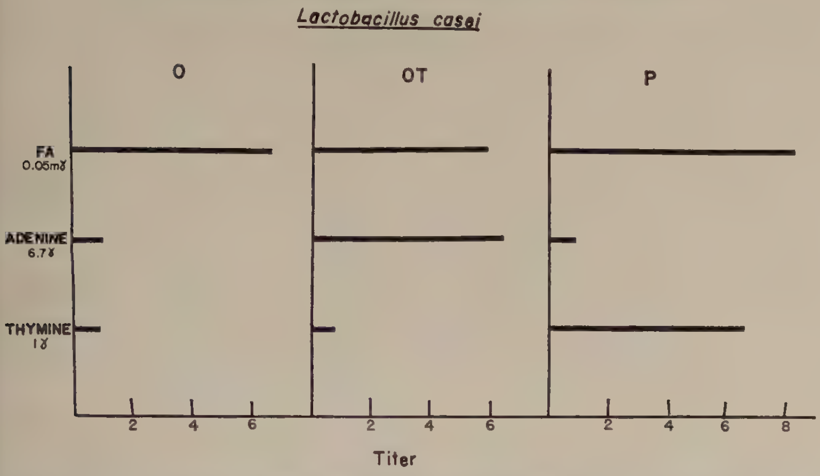

Figure 1. The effects of supplements to the basal medium on the growth of *L. casei*. O, basal medium; OT, basal medium with addition of thymine 1 γ/ml.; P, basal medium plus adenine 6.7 γ/ml. Growth is indicated by the horizontal bars (in ml. of 0.1 *N* lactic acid per 10 ml. of culture).

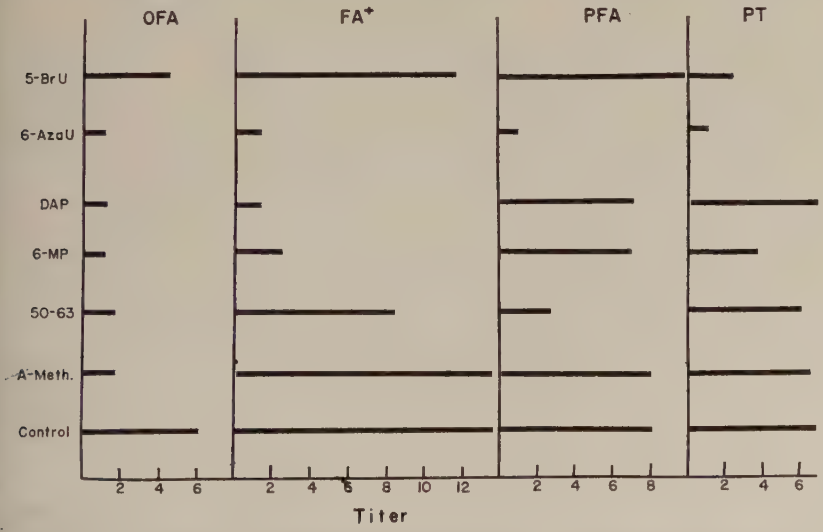

Figure 2. The effects of antagonists on the growth of *L. casei* in various media. The basal medium is supplemented with 0.05 γ/ml. folic acid to give OFA; 0.625 mγ/ml. folic acid to give FA$^+$; OFA plus 6.7 γ adenine/ml. to give PFA; OT (figure 1) plus 6.7 γ adenine to give PT. In order, the substances are 5-bromouracil, 6-azauracil, 2,6-diaminopurine, 6-mercaptopurine, pyrimethamine, and methotrexate. In each case growth is indicated by the horizontal bar (in ml. 0.1 *N* acid produced per 10 ml. of culture).

growth in both folic acid-containing media but, since the inhibition is readily reversed by the addition of purine (PFA and PT media), it is identified as a purine antagonist. 6-Mercaptopurine produces the same pattern as diaminopurine and likewise is identified on this basis as a purine antagonist. Com-

TABLE 1

STRUCTURE-ACTIVITY RELATIONSHIPS AMONG DERIVATIVES OF 6-MERCAPTOPURINE

Substituents			L. casei	S 180	
2	6	Other		Dose	Index
H	SH	—	−84	25	0.60
NH_2	SH	—	−78	4	0.60
H	S	$1\text{-}CH_3$	−15	250	0.75
NH_2	S	$1\text{-}CH_3$	−64	30	0.55
H	S	$3\text{-}CH_3$	0	125	1.00
H	SH	$7\text{-}CH_3$	0	500	0.95
H	SH	$9\text{-}CH_3$	0	500	1.00
H	SCH_3	—	−10	125	0.65
NH_2	SCH_3	—	0	125	0.60

pound 50-63 is the antimalarial pyrimethamine (Daraprim). It is identified as an antifolic acid by the inhibition in OFA medium, which is overcome in FA^+ and bypassed in the PT medium. A-Methopterin, a structural analogue of folic acid, produces a similar pattern.

The use of this test to work out structure-activity relationships is illustrated by the data of TABLE 1, in which a number of 6-mercaptopurines[15-18] are compared for activities on *L. casei*. The results of the testing of these compounds against Sarcoma 180 are included; they provide an opportunity to compare the responses of two diverse systems to these substances. It is seen that the substitution of an amino group for a hydrogen in the 2-position of 6-mercaptopurine (to produce thioguanine) increases the activity both for *L. casei* and for tumor. The 1-methyl derivatives of both 6-mercaptopurine and thioguanine show activity against S 180 when the dose is increased, and they probably retain the same type of activity for *L. casei*. This is consistent with previous findings that, of all ring alkylations, only the 1-position of the purine can be substituted without striking loss of activity. In previous work it has been found that 1-methylguanine is almost unique among substances related to guanine as a good substrate for guanase[19] and that 1-methylxanthine is oxidized by xanthine oxidase even faster than xanthine itself.[20] Substitution of the ring nitrogen in other positions, 3-, 7-, and 9-, essentially abolishes activity both for the microorganisms and for the tumor. Substitution of the mercapto group eliminates activity for the microorganism, but both the mercaptopurine and the thioguanine derivatives still have antitumor activity. One possible explanation for this difference is that the methylmercapto compounds are cleaved *in vivo* to produce the parent substances, and that this is a type of metabolic transformation that the microorganism is unable to perform. Regardless of whether this is the correct explanation for this particular case, these results illustrate the point that a one-to-one correspondence between microorganism and an *in vivo* system should not be expected.

Several years ago we participated in a survey of diverse systems for cancer chemotherapy screening sponsored by the American Cancer Society, Inc., New York, N. Y.[21] In this survey about twenty-eight agents were investigated

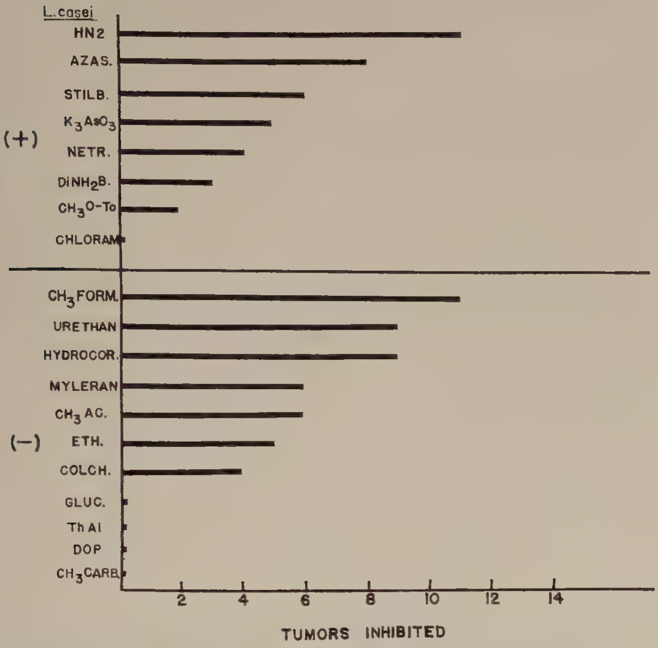

FIGURE 3. The tumor inhibitory activities of various agents showing + or − inhibitory effects on *L. casei*. In order, the substances are bis-β-chloroethylmethylamine, azaserine, diethylstilbestrol, potassium arsenite, netropsin, diaminobiuret, methoxytoluquinone, chlorambucil, methylformamide, urethan, hydrocortisone, busulfan, methylacetamide, ethionine, colchicine, D-glucosamine, β-2-thienylalanine, desoxypryridoxine, and methylcarbamate. The horizontal bars indicate the number of tumors inhibited of a possible 15.

in a wide variety of biological testing systems. Agents were selected from a group believed to have application in cancer chemotherapy and, insofar as feasible in each case, an inactive congener of the active substance was included for comparison. FIGURE 3 shows the results of testing a number of these materials in the *L. casei* screening test. In this figure the active substances are separated into two groups (above and below the horizontal line), depending on whether they did or did not inhibit *L. casei*. In each case the horizontal bar represents the number of tumor systems (of a possible fifteen) inhibited by the substance in question. The substances on this slide represent diverse agents. It is seen that *L. casei* is quite undiscriminating, and that a substance that inhibits it is no more likely to inhibit tumors than the substances that are essentially inactive against this microorganism.

However, when only antifolics and antipurines, materials for which this test was designed, are considered, a similar comparison gives a very different picture. FIGURE 4 shows the results for the materials included in Gellhorn's survey. It is seen that, of the substances in this general class, 7 would be classified as active against *L. casei*, and 1 as inactive. Using tumor inhibition as a standard of reference, *L. casei* gave 1 false positive (azaxanthine) and 1 false negative (benzimidazole). Benzimidazole is an antipurine in a

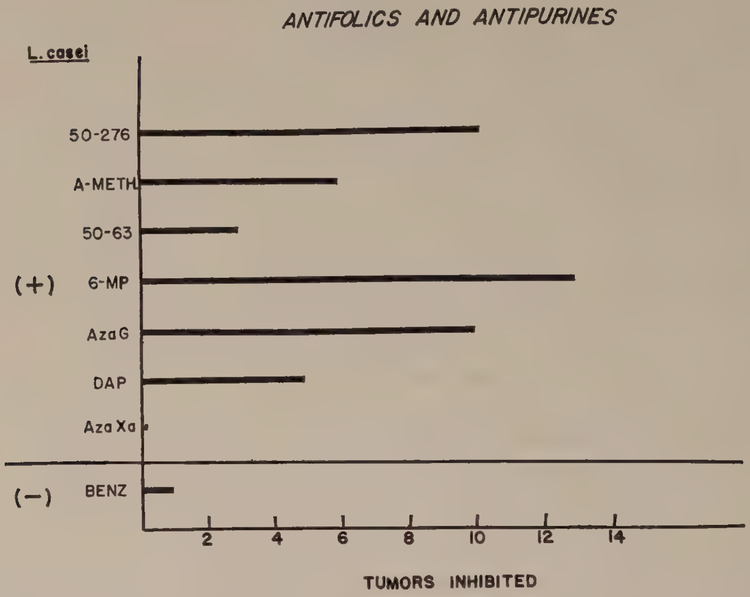

FIGURE 4. The tumor inhibitory activities of various antagonists of nucleic acid biosynthesis. In order, the substances are 2,4-diamino-5-(3′,4′-dichlorophenyl)-6-ethylpyrimidine, Methotrexate, pyrimethamine, 6-mercaptopurine, 8-azaguanine, 2,6-diaminopurine, 8-azaxanthine, and benzimidazole. The horizontal bars indicate the number of tumors inhibited of a possible 15.

number of biological systems. However, it leaves much to be desired as a tumor agent, since it produced significant effects on only 1 of the 15 tumors under consideration.

It appears, therefore, that the *L. casei* screening test is good for the purposes for which it was designed. It is capable of selecting agents of the antipurine, antipyrimidine, and antifolic acid type and, to a considerable extent, it is capable of evaluating fine details of chemical structure in relation to their effects on biological systems. Most important, perhaps, is the fact that it provides a reference biological system to which the main features of structure-activity relationships in a variety of other systems can be referred.

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Part II. Normal and Malignant Cells in Tissue Culture

INTRODUCTION TO PART II

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The reason for including a section on cell culture in a monograph presumably dedicated to screening procedures is quite simply the possibility that cell cultures perhaps provide a model system for studying the interactions between antitumor agents on the one hand and normal and malignant cells on the other. Obviously the characterization of the biological substrate is of central importance in this study. Many laboratories are attempting that characterization in terms of such factors as nutritional requirements, metabolic activity, and morphologic changes. Understandably, much of this effort has been devoted to elucidating differences between normal and malignant cells in culture, or perhaps more properly, between cells deriving from normal and malignant tissues in culture.

The basic question has not yet been resolved as to whether in these cell cultures there are constant and reproducible differences between cells deriving from normal and malignant tissues, as to whether these cultures change on serial propagation and, finally, the degree to which these changes are referable to a change in the normal and malignant characteristics of the cell.

As we shall see here, there are discrepant findings and discrepant points of view on all of these questions. We could not possibly expect the work presented here to resolve these differences, although it does, on the other hand, make the differences apparent and will serve as a stimulus to the contributors and readers alike. If we are stimulated to try to resolve these differences, then I think the program will have been worth the effort.

TUMORIGENIC ACTIVITY OF CULTURES*

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Tissue cultures of cells derived from normal and cancerous sources have been studied for their ability to produce tumors. The object of the study has been twofold: (1) to learn whether they still produce tumors, and (2) if so, whether the tumors produced are histologically like the original tumor. Since the cell lines used[1] are derived from human material, one of the test animals employed has been the irradiated and cortisone-treated weanling rat prepared according to the Toolan method.[2] Cell lines also have been tested for their ability to grow in the embryonated egg.[3] Occasionally, in connection with another study, human volunteers with cancer also have been inoculated.[4] The results constitute the subject of this paper.

Rat Inoculations

In addition to testing established lines of cells in tissue culture, we made numerous attempts to grow tumors received directly from the operating room by mincing them and allowing them to grow for a time before inoculation into the rat. It was hoped that enough cancer tissue would be freed from its stroma so that the cancer cells might form a tumor when inoculated. Thirteen breast cancers and 11 cancers from miscellaneous sources failed to grow. Normal tissue grown from trypsinized human kidney and testicle also failed to grow in 4 instances, as did 8 human adult and embryonic fibroblast cultures which, in 1 instance, had been kept in culture for 3 months with 16 trypsinizations before it was lost by contamination. Our usual experience has been that human fibroblast cultures grow readily at first, but die out after 6 or 7 trypsinizations.

Since the time of isolation in tissue culture, it has been routine to inoculate 2 or 3 milk-dilution bottles containing a total of 3 to 5 million cells subcutaneously into prepared rats. When tumors formed they were removed 10 to 14 days later for histological examination. TABLE 1 gives the results and shows that cell lines well established in tissue culture produce tumors in the majority of inoculations.

In another type of experiment, counted cells were inoculated subcutaneously to determine if the number necessary for tumor production was the same for each tumor. TABLE 2 summarizes these results. In the final column is listed the percentage of inoculations that produced tumors when 1 million or more cells were inoculated. The cell lines HEp 3 and HS 1, which are well adapted to rat passage and grow well on the chorioallantoic membrane of embryonated eggs,[3] give the highest percentage of tumors. The Osgood J-111 strain,[5] which came originally from a monocytic leukemia, also produced a

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* The work reported in this paper was supported in part by a research grant from the National Cancer Institute, Public Health Service, Bethesda, Md.

Table 1

Production of Tumors in the Prepared Rat with Established Tissue Culture Cell Lines

Cell line	Tumors produced/No. inoculations
HEp 1	5/5
HEp 2	5/8
HEp 3	8/9
HeLa	3/4
J-111	12/12

Table 2

Production of Tumors in Relation to the Number of Cells Inoculated

Cell line	Number of cells inoculated							Percentage of tumors
	<500T	500T–1M	1–3M	3–5M	5–10M	10–20M	>20M	
	Tumors produced/No. inoculations							
HEp 1	0/4	3/8	3/5	2/2	2/2	2/4	0/3	56
HEp 2	0/4	0/4	6/12	1/8	2/2		1/1	43
HEp 3	12/17	1/3	24/29	30/38	1/2			80
HS 1		1/12	55/61	13/20	1/1			84
HeLa*	0/1			1/12	6/16	12/17	4/4	47
J-111			1/1	2/2	0/1	2/4	8/8	81
Chang lines†	0/2	0/3	2/6	2/5	5/6	0/1		50
Fl amnion		0/2	0/2	2/2	1/2			50

Symbols: T = thousand; M = million.
* Puck's strains.
† Kidney and conjunctiva.

high percentage of tumors; in at least one instance following intraperitoneal inoculation, tumor implants were found in the liver (FIGURE 1). However, this cell line appears to be much less invasive in the human volunteer cancer patient. The other cell lines produce tumors less efficiently. Both cell lines derived from normal sources[6] that have altered during their cultivation in tissue culture[7] give about 50 per cent tumors, as do cloned HeLa cells and the HEp 1 and HEp 2 cell lines.

The HeLa cell lines reported were received through the kindness of Theodore Puck and are cloned nutritional and X-ray mutants;[8] therefore the results of their inoculation may not be the same as for the usual uncloned HeLa. The lines studied were SI, S3RA1, S3R3, S3RE1, S3RB1, S3RS, and S3R9, and tumors were obtained with each cell line. There seems to be a difference in the numbers of cells necessary to produce a tumor, but more work is necessary to ascertain if this is more than an accidental finding. The tumors formed, although showing minor histological differences, are of the same type, although in some instances their morphologic appearance in the culture is quite different.

A comparison of different routes of inoculation was made by the inoculation

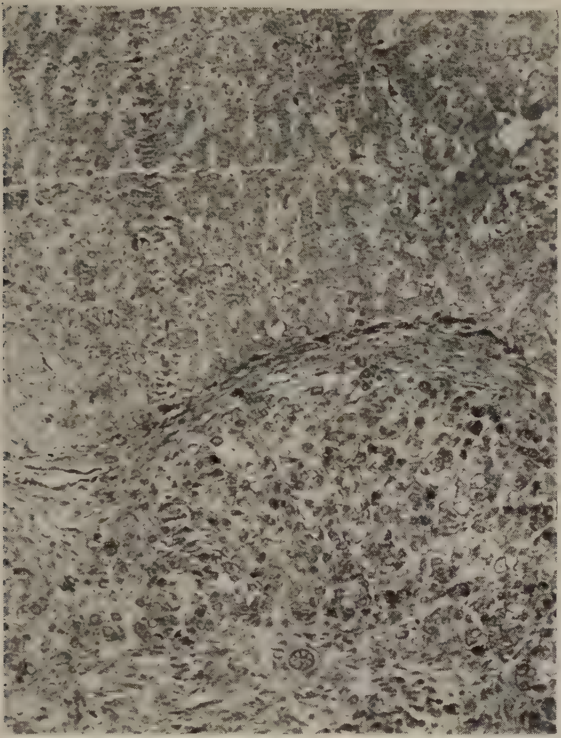

FIGURE 1. Tumor implant on the liver surface in a conditioned rat inoculated intraperitoneally with 100,000,000 J-111 cells. Nodules also were present on the diaphragm, kidney, spleen, and mesentery.

of 500,000, 1 million, and 5 million HEp 3 cells, subcutaneously, intraperitoneally, and intramuscularly in prepared mice[9] and rats.[2] The inoculation of 5 million cells resulted in production of tumors in all animals. With less cells there was no great difference between the number of tumors produced, but the intramuscular site proved best for mice. The tumors averaged 2.7 gm. in weight and were 4 times the weight of those obtained by the subcutaneous route. These findings are in agreement with those of other workers.[10]

In all work with irradiated and cortisonized rats it must be remembered that there may be considerable variation in the response of the animal itself. There is also a difference in the way the animal responds to conditioning, which may influence the results.

Egg Inoculations

Most cell lines also have been investigated for their ability to produce tumors in eggs. Different methods of inoculation have been tried, such as imbedding in a plasma clot and planting near a blood vessel in the same way in which tumor pieces are implanted, as well as the more conventional yolk sac and

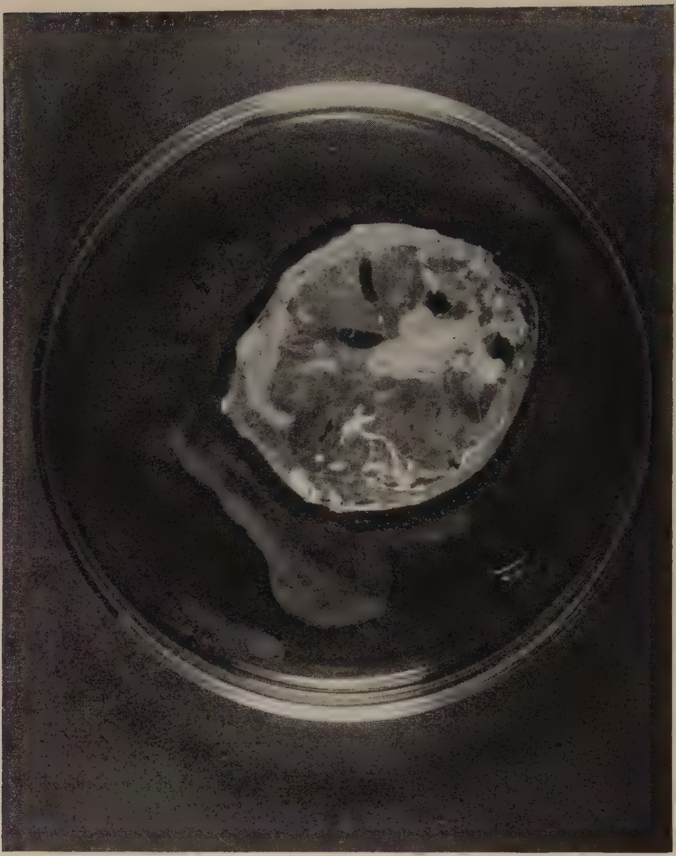

FIGURE 2. Large and small tumor nodules that appeared on the chorioallantoic membrane of a 9-day-old embryonated egg 10 days following the inoculation of 1,000,000 HEp 2 cells.

chorioallantoic membrane inoculations. With 1 or 2 exceptions, only the chorioallantoic membrane method (in which approximately 500,000 to 1 million cells suspended in 0.2 cc. of media were allowed to run over the membrane) produced tumors. The type of growth that resulted is illustrated in FIGURE 2 and shows the solid tumors that form, as well as the nodules that appear along the vessels. The results listed in TABLE 3 are separated according to the age of the egg when inoculated, since it is apparent that this is an important factor. The cell lines varied considerably in their ability to produce tumors, HEp 3 being by far the most efficient, whereas the Chang cell lines failed to produce any tumors. HEp 2 produced tumors quite readily, while HEp 1 and HeLa did poorly. A titration done with HEp 3 showed that tumors could be produced regularly by the inoculation of 250,000 cells, whereas the other cell lines required many more.

TABLE 3

RESULTS OF INOCULATION OF ESTABLISHED CELL LINES ON THE CHORIOALLANTOIC MEMBRANE OF FERTILE EGGS

Cell line	Tumors/inoculations		Percentage of tumors produced
	7-day eggs	>7-day eggs	
HEp 1	0/17	7/23	30
HEp 2	7/39	13/19	68
HEp 3	Not done	9/9	100
HeLa	2/22	3/14	21
Chang lines*	0/19	0/13	0

* Liver and conjunctiva.

Inoculation of Cancer Patient Volunteers

It is difficult to make direct comparisons of the degree of malignancy in human volunteers with cancer, since in most instances the nodules are removed within two weeks after inoculation. However, a certain number have been left *in situ*, and their rate of regression, along with the reappearance of a new

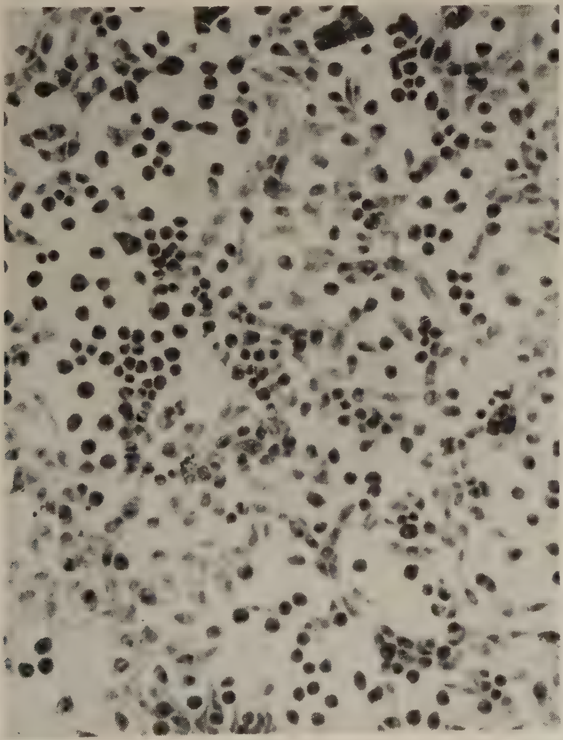

FIGURE 3. HEp 1, showing the mosaiclike appearance and round cells that characterized its appearance when it was first isolated. May-Grünwald stain. ×160.

tumor at the biopsy site, allows some measurement of their relative growth abilities. The observations, made by Southam,[4] who is in charge of this work, are as follows:

Tumor production. Inoculation of HEp 1, HEp 2, HEp 3, HeLa, and J-111 has always resulted in the appearance of a tumor at the site of inoculation, except on one occasion with HeLa and on another with J-111. Inoculation of the Chang cell lines resulted in the production of epithelial growths in 7 of 10 instances, and the inoculation of the Fl amnion cells[11] produced tumors in 6 of 6 instances. Inoculation of first generation amnion (3 instances) and cultured fibroblasts (4 instances) never resulted in tumors.

Regressions. Almost all tumors not removed by biopsy began to regress within a month, with the striking exception of HEp 3, which grew progressively in every instance. On one occasion the Fl amnion continued to grow slowly until the death of the patient 38 days later.

Recurrence at biopsy site. In the great majority of recurrences, the cell line inoculated was HEp 3. However, there was a recurrence of one each with HEp 1, HEp 2, Chang conjunctiva, and Fl amnion.

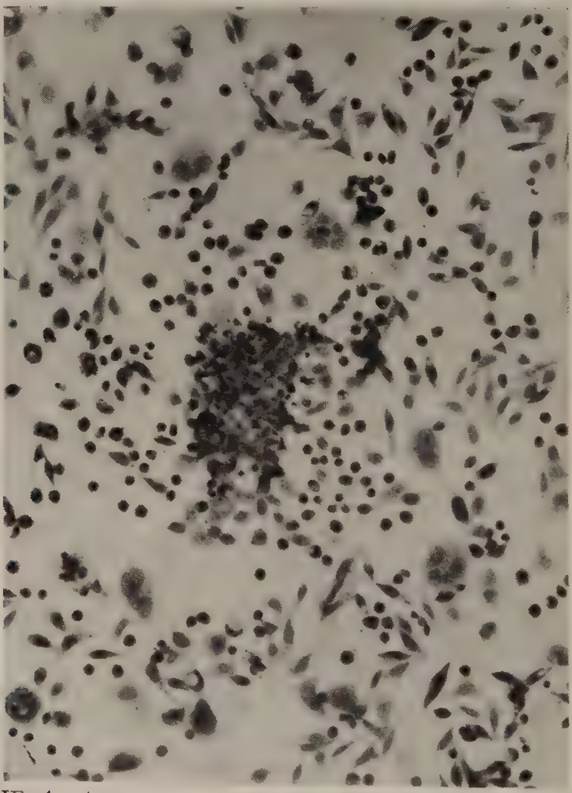

FIGURE 4. HEp 1. Appearance of cells derived from a single cell using Puck's technique and showing the great variety of cells found. May-Grünwald stain. ×160.

There seems to be little doubt that the growth potential of the HEp 3 cell in the human volunteer cancer patients is much greater than that of the other cell lines. The J-111 cell line and the Chang cell line appear to be at the other end of the scale, with the other cell lines ranged in between. Here, too, variation from patient to patient must be taken into account.

Stability of Tissue Culture Cell Lines

The cell lines that have been discussed have retained their same morphologic appearance and produced the same type of tumor as seen in the original patient, with the exception of HEp 1, which was observed to contain many more bizarre forms than when it was first isolated five years ago. At that time the predominant cells were rather angular ones that fit together to form a mosaic and contained quite a few round cells thought to be rounding up prior to mitosis. Giant cells and multinucleated cells were rare to absent. The cell line retained this form until a year ago, when many odd forms were noted in the cultures. Recently, cloned cell lines have been attained by Leila Diamond, using Puck's method; FIGURES 3, 4, 5, and 6 demonstrate the type

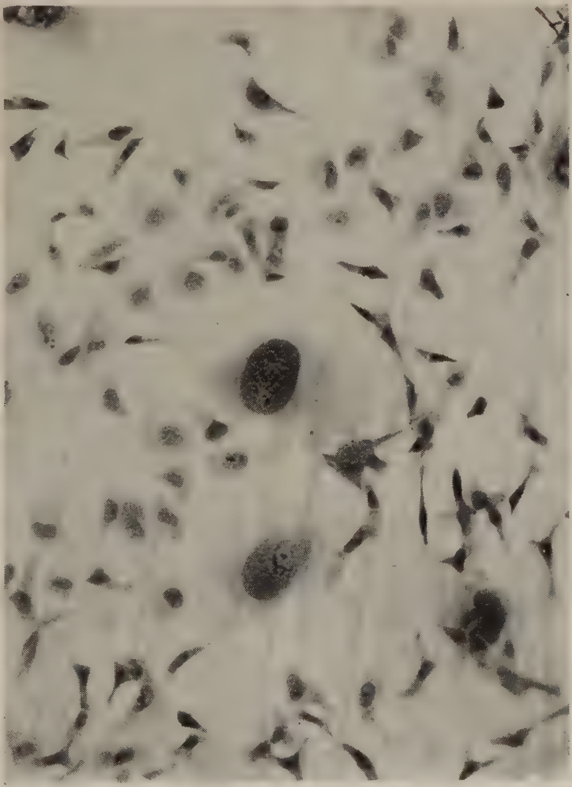

FIGURE 5. HEp 1. A cloned cell line demonstrating the much larger cells that occur and the single giant cells that occur. May-Grünwald stain. ×160.

of cells that have become prominent in the cultures. FIGURE 3 shows the appearance of the original culture; FIGURE 4, one cloned line in which the small cells that originally composed the main part of the culture are still present, along with some of the variations; FIGURE 5, the huge single giant cells that occur; and FIGURE 6, the bizarre multinucleated cells now found. As yet, not enough cloned cells have been available to inoculate into treated animals, especially since the large multinucleated cell line grows very slowly. However, inoculation of the stock cultures from which these arose still produces tumors that are histologically like the original tumors, although one gets the impression that the percentage of "takes" has decreased somewhat from that obtained when the culture was freshly isolated.

A very curious happening also may be recorded as an instance of the care that must be taken to keep one's cultures in order. On two different occasions a human embryonic rhabdomyosarcoma, HEmbRh 1,[1] which grows in a very peculiar fashion in tissue culture, developed a more or less fibrotic form that, when reinoculated into a treated rat, produced a tumor that did not resemble the original tumor and that, on subsequent transplantation into nontreated

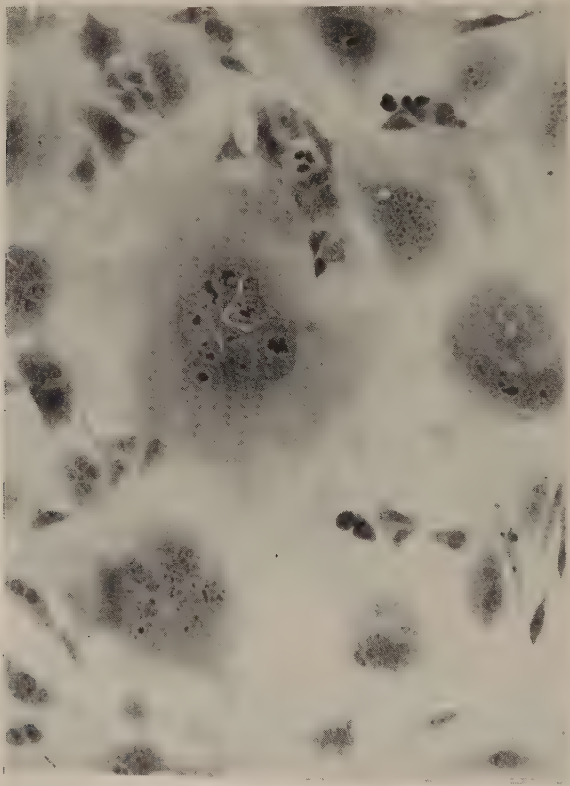

FIGURE 6. HEp 1. A cloned cell line, demonstrating the bizarre multinucleated cells. May-Grünwald stain. ×160.

rats, produced a sarcoma. An examination of its chromosomes by Levan[12] confirmed the fact that it was indeed a rat tumor. We have not been able to repeat this phenomenon in at least twenty attempts and have no real explanation for it. It can be speculated that the human tumor died out and was replaced by a rat stroma that altered during its *in vitro* cultivation.

Summary

In summarizing our results of inoculating established cell lines into different hosts, it can be stated that those lines originating from cancer are capable of producing a tumor of the same histological type, and that the Chang cell lines from normal sources that have altered during culture show the same ability, but to a lesser degree. The Fl amnion cell line appears to be much more vigorous than the Chang cells in producing tumors in man. What few established human fibroblast cultures we have been able to test produced no tumors in any host. As might be expected, the volunteer cancer patient is the best host, but certainly the irradiated and cortisonized rat gives satisfactory repeatable results and is therefore the choice test animal. Because of the low percentage of takes, the chorioallantoic membrane does not appear to be useful as a test for tumorigenicity.

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TUMORIGENIC ACTIVITY OF TISSUE CELL CULTURES*

By G. E. Foley and A. H. Handler

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The fact that cells derived from malignant and nonmalignant sources are difficult to distinguish from one another after cultivation *in vitro* has become more or less accepted by those experienced in such matters; thus far, comparative studies have failed to reveal constant, significant morphologic or biochemical differences between cells of malignant and nonmalignant origin in tissue cultures. The similarity in the biochemical behavior of such cells, even in well-defined substrates,[1-7] is emphasized in FIGURE 1 (see also TABLE 1), which is taken from some recent studies[8] on the response of cells of different origins to various antimetabolites in tissue culture. In this figure, the various antimetabolites are arranged on the abscissa in decreasing order of activity, and the cytotoxicity of each for four cell lines of malignant and three cell lines of normal origin is indicated on the ordinate. It is evident that, with respect to a given inhibitor, all cell lines tested behaved similarly.

Obviously, this sort of observation is in direct conflict with the selective antitumor activity exhibited by many of these compounds *in vivo*. At first glance, the explanation is equally obvious: either the selective antitumor activity observed *in vivo* is host imposed, or all cells in tissue culture become "malignant." The opposite view of the latter possibility, that is, that all cells in tissue culture become "normal," is equally plausible and equally without objective evidence, but it seems to have attracted little attention.

Cells cultivated in Eagle's medium[1-7] or variations thereof attach directly on glass without the necessity of an intervening matrix, and they grow in an essentially monocellular layer, approaching the growth rate of some of the higher bacteria and fungi. Preparation of suspensions of single cells from such cultures, which can be quantitated directly, is a relatively simple matter; in experiments with Sarcoma 180[9] it was observed that, when quantitated inocula were implanted in CFW mice, a typical log-dose-response relationship was obtained.

These experiments with S 180 in mice suggested that human tissue culture cell lines of malignant origin might be titrated similarly by heterotransplantation to the cheek pouches of the golden hamster (*Mesocricetus auratus*), and that those cell lines derived from nonmalignant sources might behave differently, since it is well known that the hamster accepts heterotransplants of malignant tissue,[10, 11] but rejects heterotransplants of normal (adult) tissue.[11]

Following the observation that the KB[12] and HeLa[13] cell lines derived from human epidermoid carcinomas could be titrated in the hamster cheek pouch,[14] a study of all of the available tissue culture cell lines was undertaken in the

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TABLE 1

COMPOUNDS STUDIED FOR CYTOTOXICITY IN TISSUE CULTURE*

Compound No.	Trivial name	Lot number	Source of sample†	Chemical name
1	Aminopterin	7-7491	1	4-Aminopteroylglutamic acid
2	A-Methopterin	7-8528	1	4-Amino-N^{10}-methylpteroylglutamic acid
3	Bremfol	7-8942	1	Pteroyl-9-methylglutamic acid
4	Amino alanfol	7-8514	1	4-Aminopteroyl-DL-α-alanine
5	An-fol-A	7-7469A	1	Pteroylaspartic acid
6	Amino anfol	7-8686	1	4-Aminopteroylaspartic acid
7	Azaserine	236X34-6V	2	*o*-Diazoacetyl-L-serine
8	Stylomycin	7-8340	1	6-Dimethylamino-9-(3′-*p*-methoxy-L-phenylalanylamino-3′-deoxy-β-D-ribosyl)purine
9	6-MP	AN32187	3	6-Mercaptopurine
10	JF-5	1687-C-41A	1	Actinomycin D
11	TEM	7-5440	1	N',N',N''-Triethylenemelamine
12	Thio-TEPA	7-8940	1	N',N',N''-Triethylenethiophosphoramide
13	Daraprim	50-63	3	2,4-Diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine
14	D-54·HCl	PL 161 I/C	4	4,6-Diamino-1-(3′,4′-dichlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine
15	D-69·HCl	HK 31 B/B	4	4,6-Diamino-1-(*m*-chlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine

* Reproduced from Eagle and Foley[8] by permission of *The American Journal of Medicine.*

† Symbols: 1, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.; 2, Parke, Davis & Co., Detroit, Mich.; 3, Burroughs Wellcome Laboratories, Tuckahoe, N. Y.; 4, The Children's Cancer Research Foundation, Boston, Mass.

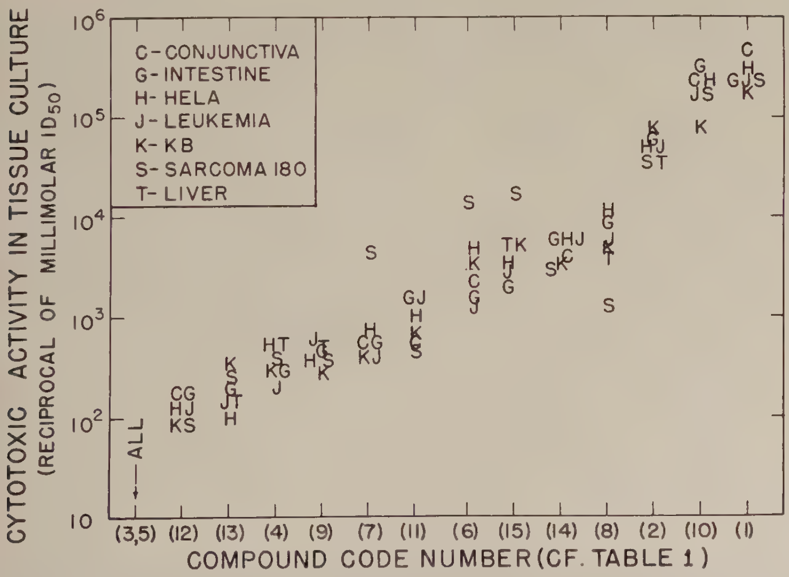

FIGURE 1. Essentially uniform susceptibility of seven normal and malignant cell lines to a number of carcinolytic agents. Reproduced from Eagle and Foley[8] by permission of *The American Journal of Medicine.*

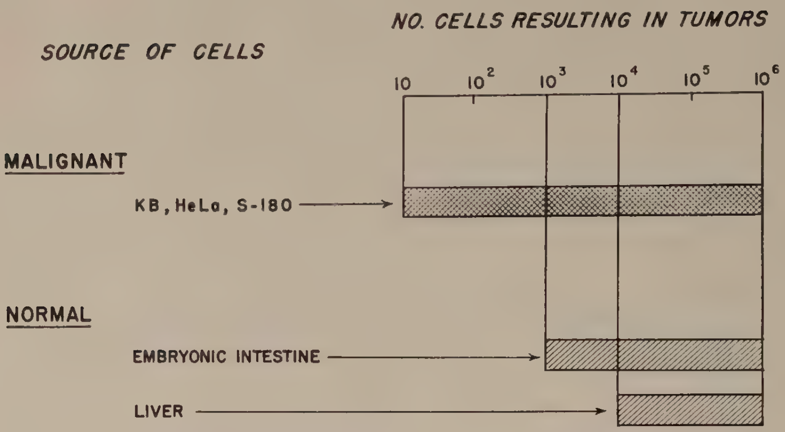

FIGURE 2. Titration of tissue culture cell lines in cortisone-treated hamsters.

hope that those of normal origin, like normal tissue, might fail to grow in this heterologous host.

The techniques employed in these studies have been described elsewhere[14, 15] and will not be discussed here, except to mention the fact that the hamsters were prepared for implantation as described by Lutz *et al.*,[16] and that, in those instances where cortisone was used, it was administered in subcutaneous doses of 2 to 3 mg. simultaneously with implantation and twice weekly postimplantation.[17]

In preliminary experiments (FIGURE 2) with cortisone-conditioned hamsters,[15] all cell lines grew if the inoculum contained 1.0 × 10⁶ or more cells; however,

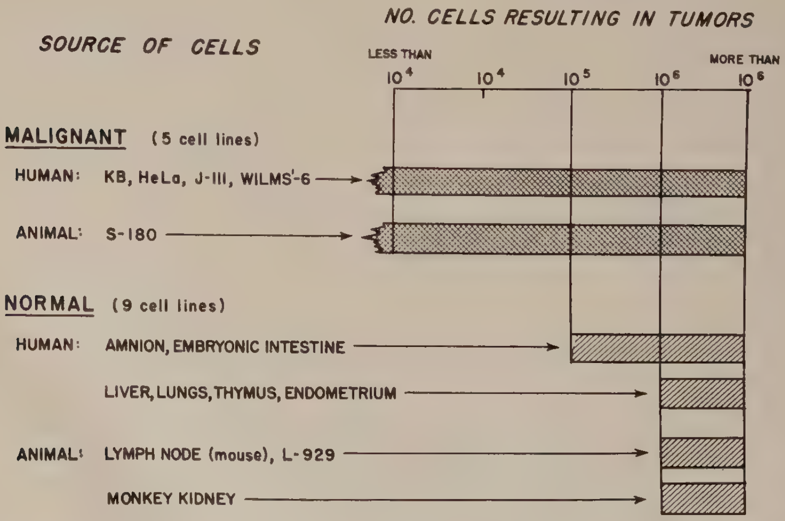

FIGURE 3. Titration of tissue culture cell lines in normal hamsters.

cell lines derived from malignant sources produced tumors following implantation of approximately 10 cells, while those of normal origin failed to survive and grow if the inoculum contained fewer than 1000 cells. This difference (FIGURE 3) was even more pronounced when normal unconditioned hamsters were used.[15] Again, cell lines derived from malignant sources produced tumors following implantation of 1000 or fewer (in many instances, as few as 10) cells, while cell lines derived from adult nonmalignant sources failed to survive and grow if the inoculum contained less than 100,000 to 1,000,000 cells. Cell lines of normal embryonal origin appear to be intermediate between those of malignant and adult normal origin in their ability to survive and grow in the hamster cheek pouch (FIGURE 3). It is of interest to call attention to the marked difference in the behavior of, for example, HeLa and normal liver or embryonic intestine tissue culture cell lines in the hamster cheek pouch (FIGURE 3). As indicated in FIGURE 1, these cell lines thus far cannot be differentiated on the basis of their response to antimetabolites *in vitro*.

Additional differences in the behavior of cell lines of normal and malignant origin in the hamster cheek pouch also are apparent; namely, unlike the frankly invasive, transplantable tumor produced by cell lines of malignant origin, the cell growth resulting from large inocula (100,000 or more cells) of cell lines of normal origin is: (1) not invasive, (2) regresses rapidly, and (3) cannot be transplanted to other hamsters. The characteristic histological differences exhibited in the hamster cheek pouch by implants of cell lines of malignant and normal origin may be illustrated (FIGURE 4) by comparison of the Osgood J-111* cell line isolated from the peripheral blood of an adult patient with acute monocytic leukemia,[18] and the Detroit 173B† cell line isolated from the peripheral blood of a normal, healthy adult.[19] Inocula of 1.0×10^5 cells of either cell line, harvested from 5-day-old *in vitro* cultures in defined media, were implanted in the cheek pouches of cortisone-conditioned hamsters, which were sacrificed for histological study on the ninth postimplantation day.

The implants of malignant cells (Osgood J-111) were growing vigorously, as evidenced by marked mitotic activity (FIGURE 4a), and had invaded the surrounding host tissue. Histological sections showed some necrobiosis, but no necrosis or invasion of the growing implant by host inflammatory cells. On the other hand, sections of cheek pouches implanted with the normal cell line (Detroit 173B, FIGURE 4b) indicate that these implants did not invade the surrounding host tissue, and exhibited only an occasional cell in mitosis. These implants were diffusely infiltrated by host inflammatory cells, and considerable host inflammatory reaction surrounded what remained of the original implant. Coriell *et al.*[20] have reported similar differences in the behavior of tissue culture cell lines of malignant and normal origin in experimental animals.

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* Obtained through the courtesy of Harry Eagle, National Institute of Allergy and Infectious Diseases, Public Health Service, Bethesda, Maryland.

† Obtained through the courtesy of C. S. Stulberg, The Child Research Center of Michigan, Detroit, Mich.

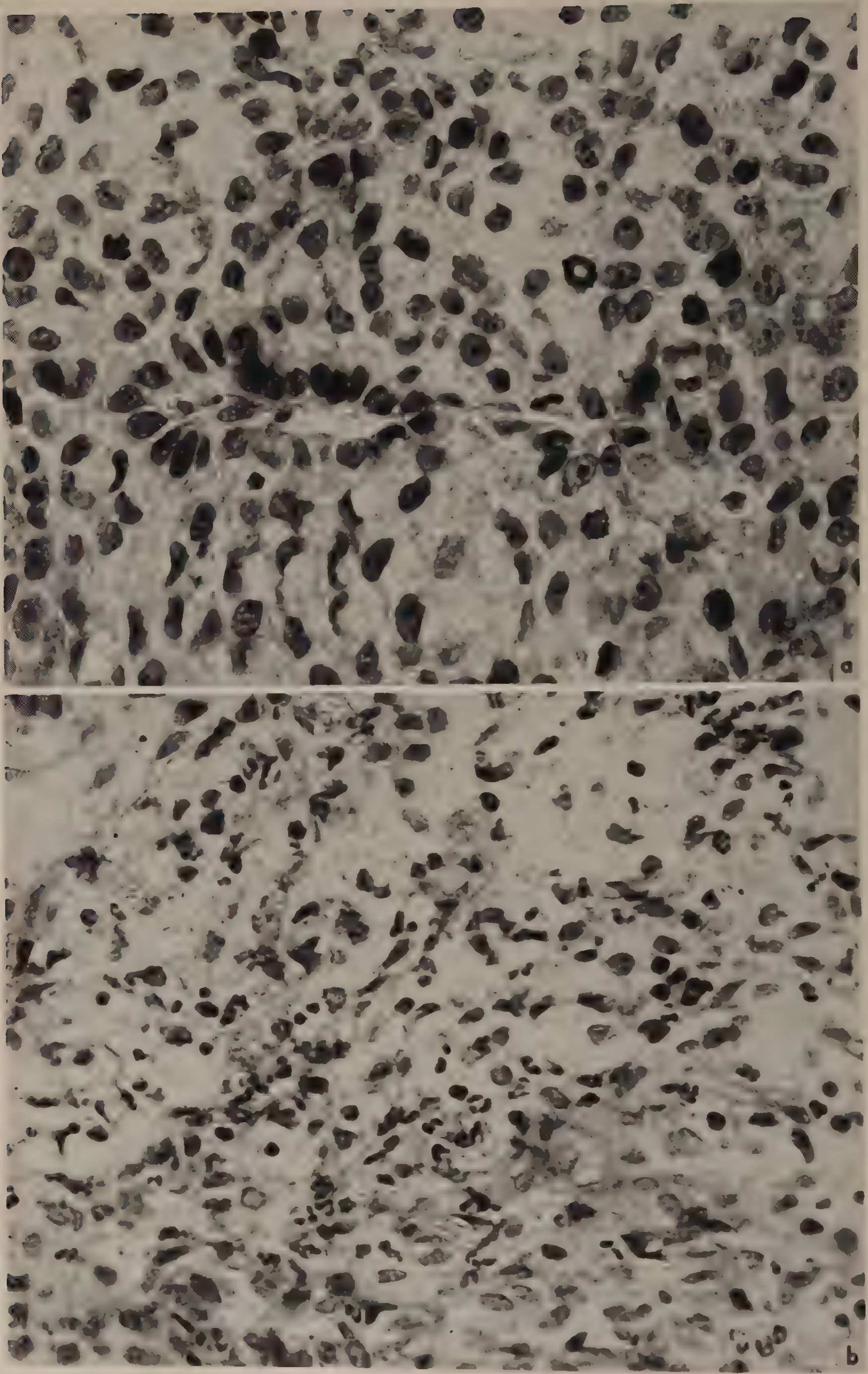

FIGURE 4. Histological differences exhibited by tissue culture cell lines of malignant and normal origin implanted in the hamster cheek pouch. (a) Osgood J-111, isolated from peripheral blood of an adult with acute monocytic leukemia and sectioned on the ninth day following implantation of 1.0×10^5 cells. H & E. ×500. (b) Detroit 173B, isolated from peripheral blood of a normal healthy adult and sectioned on the ninth day following implantation of 1.0×10^5 cells. H & E. ×500.

These transplantation studies are still in progress; to date twenty additional cell lines isolated in our laboratories during the past two years, as well as fifteen cell lines obtained from other investigators have been or are being examined. The results thus far are consistent with the difference between cell lines of malignant and normal origin as herein described, with one exception, a cell line (Detroit 52)* isolated from the bone marrow of a patient with diabetes.[21] The behavior of this cell line has consistently resembled that of embryonal cells in the hamster cheek pouch, that is, intermediate between frankly malignant and frankly normal behavior.

Discussion

As yet, there is no factual explanation for this difference in the "growth potential" of cell lines of malignant and normal origin. It has been said that studies such as these merely titrate differing "degrees of malignancy"—a comforting explanation for those who wish to accept the hypothesis that all cells in tissue culture are malignant. However, if such were actually the case, it would be difficult to explain the observed correlation between the source of the cell line and the capacity of small inocula to survive and grow in the cheek pouch. A more likely explanation is that these differences in growth potential represent inherent differences in capacity for autonomous growth in this heterologous host. A similar difference between the capacities of malignant (an adenomatous gastric lesion of mice) and normal (mouse gastric mucosa) tissue to grow when titrated in homologous and heterologous mice was recorded as long ago as 1941 by Andervont and Shimkin.[22] There is an obvious similarity between these observations with tissue culture cells and the conclusions reached by Greene[23] on heterotransplantability as a criterion for malignancy.

The immediate significance of the fact that the hamster differentiates between tissue culture cell lines of normal and malignant origin does not lie in the realm of another biological definition of malignancy, but rather in the idea that this observed biological difference suggests the possible existence of related biochemical attributes, despite the lack of knowledge of any such biochemical distinctions at the present time.

Summary

Recent studies indicate that the majority of recognized antitumor agents are equally cytotoxic in tissue culture against cells deriving from normal and tumor tissue. This paradox, together with other *in vitro* biochemical and morphologic similarities between cell lines derived from normal and tumor tissue, suggests that cell lines of normal origin are no longer normal, but have assumed, as a consequence of *in vitro* cultivation, some of the characteristics usually attributed to malignant cells.

However, despite these similarities, definite biological differences between cell lines derived from malignant and normal tissue have been delineated by

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* Obtained through the courtesy of C. S. Stulberg, The Child Research Center of Michigan, Detroit, Mich.

titration in the cheek pouch of cortisone-conditioned and unconditioned Syrian hamsters (*Mesocricetus auratus*).

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COMPARISON OF BIOLOGICAL QUALITIES OF "TRANSFORMED" CELLS OF NORMAL AND CANCEROUS ORIGIN*

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Among cancer workers the emergence of continuous lines of human cells has given rise to creative speculation in many areas of interest—carcinogenesis, nuclear division, transplantation, immunity, and chemotherapy, to name but a few. In our laboratory the cell lines are providing an interesting tool with which to explore *in vitro* and *in vivo* the interactions between microscopically normal and malignant cells. In experimental systems that enable us to examine the cancerlike behavior of different cell lines, variations in their biological qualities are seen. This paper describes some of our observations, with emphasis on the features that may be of use in a biological classification of cell lines.

The interaction of Gey's HeLa cells with normal tissues of human and of chick embryonic origin in sponge-matrix tissue culture has been described.[1-3] In general, normal tissues that give rise to connective tissue *in vitro* are readily invaded and progressively replaced by HeLa cells. Two other cell lines of cancerous origin (Eagle's KB and Osgood's J-96) have been seen to invade a variety of human fetal connective tissues *in vitro* in much the same manner as HeLa. The patterns of interaction seen in these combinations, as complicated by the organized epithelial elements of fetal host tissues, will be described in a subsequent paper.

The development of continuous lines of cells derived from normal tissue appeared to afford an opportunity to compare the behavior of cancer cells and normal cells. On microscopic examination of several continuous lines of "normal" cells, however, all of the cytological features of cancer cells were seen. This suggested that, rather than being normal, rapidly growing cell lines of normal derivation had themselves undergone cancerous transformation.[4-6] The experience of several observers using varied test systems in the past year or two attests to the reasonableness of this interpretation.[7-9] Our experience with lines of "normal" cells also is consistent with this interpretation.

Observations

In a preliminary trial, in which several cell lines were placed on cultures of normal-appearing fibroblasts growing on glass, distinct differences in the degree of spread of the continuous cell lines were observed. Chang's Conjunctiva and our D-189, both of which were derived from normal tissue, seemed to be at the two extremes of invasive spread, with HeLa and KB somewhere in between.

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TABLE 1
DATA ON COMPARISON OF CELL LINES IN TISSUE CULTURE

Cell line	Host tissue	Type of culture	Number of cultures	Days in combination	Composition of medium*
HeLa	12 different human tissues	Sponge matrix	12 (1 for each host tissue)	11 days	30 % human serum 25 % beef amniotic fluid 3 % beef embryo extract
KB			12 (1 for each host tissue)	11 days	
J-96			12 (1 for each host tissue)	11 days	
HeLa	Human fetal connective tissue (Microbiological Associates)	Sponge matrix	2	13 days	30 % human serum 25 % beef amniotic fluid 3 % beef embryo extract
KB			2	13 days	
D-189			2	13 days	
D-189	Connective tissue from the skin of a 9-day-old chick embryo	Sponge matrix	5	7 days	20 % human serum 5 % beef embryo extract in Eagle's basal medium
Conjunctiva			5	7 days	
D-189	Umbilical cord; frozen & thawed	Cell suspension inoculated into pieces of cord	4	7, 20, 23, 23 days	10 % human serum in Eagle's basal medium
Conjunctiva			4	16, 20, 37, 37 days	
D-189	Umbilical cord; frozen & thawed	Gelfoam fragments implanted into pieces of cord	9	16 & 24 days	10 % human serum in Eagle's basal medium
Conjunctiva			10	16 & 24 days	
D-189	Microbiological Associates Human Fibroblasts (MAF)	Agar overlay	2	15 days	20 % human serum 5 % beef embryo extract in Eagle's basal medium
Conjunctiva			2	15 days	
D-189	Connective tissue from the heart of an 18-day-old chick embryo	Agar overlay	14	9 days	20 % human serum 5 % beef embryo extract in Eagle's basal medium
Conjunctiva			7	9 days	
HeLa			7	9 days	

* All media contain 50 μg. of streptomycin and penicillin (Microbiological Associates, Inc., Bethesda, Md., mixture) and are made up in Hanks' balanced salt solution.

That a "normal" cell might be more invasive than HeLa or KB seemed unlikely, and the cell lines D-189, KB, and HeLa were compared more critically, using sponge matrix (TABLE 1). Examination of serial sections of these cultures clearly demonstrated that D-189 had capacities for invasive spread that were greater than the capacities of either HeLa or KB. D-189 cells were found in the connective tissue at great distances from the site of implantation (FIGURE 1). Comparable distant spread was very uncommon in the two cancer cell lines. In sponge-matrix tissue culture systems then, cell lines of normal origin could be more invasive than cells of cancerous origin.

In another experiment, D-189 was found to be much more invasive than Conjunctiva (FIGURE 2). We then compared these two lines of normal origin, D-189 and Conjunctiva, in tissue culture systems designed to present the "transformed" cells with different problems. Two systems currently in use for making these comparisons are implantation into umbilical cord and agar-overlay methods. In addition, cell lines are being compared *in vivo* by direct inoculation onto the chick chorioallantoic membrane, and by inoculation on homologous human skin, which is then placed on the chorioallantoic membrane ("companion hosts").

Umbilical Cord Matrix

An examination of the invasive capacity of "transformed" cells implanted in a nonviable connective tissue provides an opportunity to compare the patterns of tumor spread that are intrinsic to the cells, uncomplicated by the possible responses of living host tissue. An umbilical cord from a 6-month-old miscarriage was frozen; pieces were cut from it and thawed to provide the nonviable connective tissue matrix. In one experiment a suspension of each of the cell lines was injected into pieces of cord. In another, Gelfoam particles containing the cells were implanted, with the aid of a trocar, into pieces of cord. The cord fragments varied considerably in size, ranging from 1.0 to 0.5 cm. in each dimension. Each culture was prepared by placing several small pieces or one large piece in a tube with nutrient medium (TABLE 1). The cultures were kept in a roller drum at 35° to 37° C.

Both cell lines gave evidence of invasion from the sites of Gelfoam implantation when serial sections were examined (FIGURE 3a). However, D-189 had spread out from the Gelfoam inocula to a much greater extent than had the Conjunctiva cells. An interesting feature of both cell lines was the arrangement of the cells. They were usually seen in oval or round aggregates. The microscopic picture that each presented is one that is generally considered characteristic of cells of epithelial derivation. This is of interest, since D-189 was derived from connective tissue cells (FIGURE 3b).

Patterns of aggregate growth were not seen at all in instances where cells of either line were found on the surface of the cord fragments. In these sites (just as on the surface of connective tissue cultures in sponge matrix) the cell lines failed to appear in easily defined groupings.

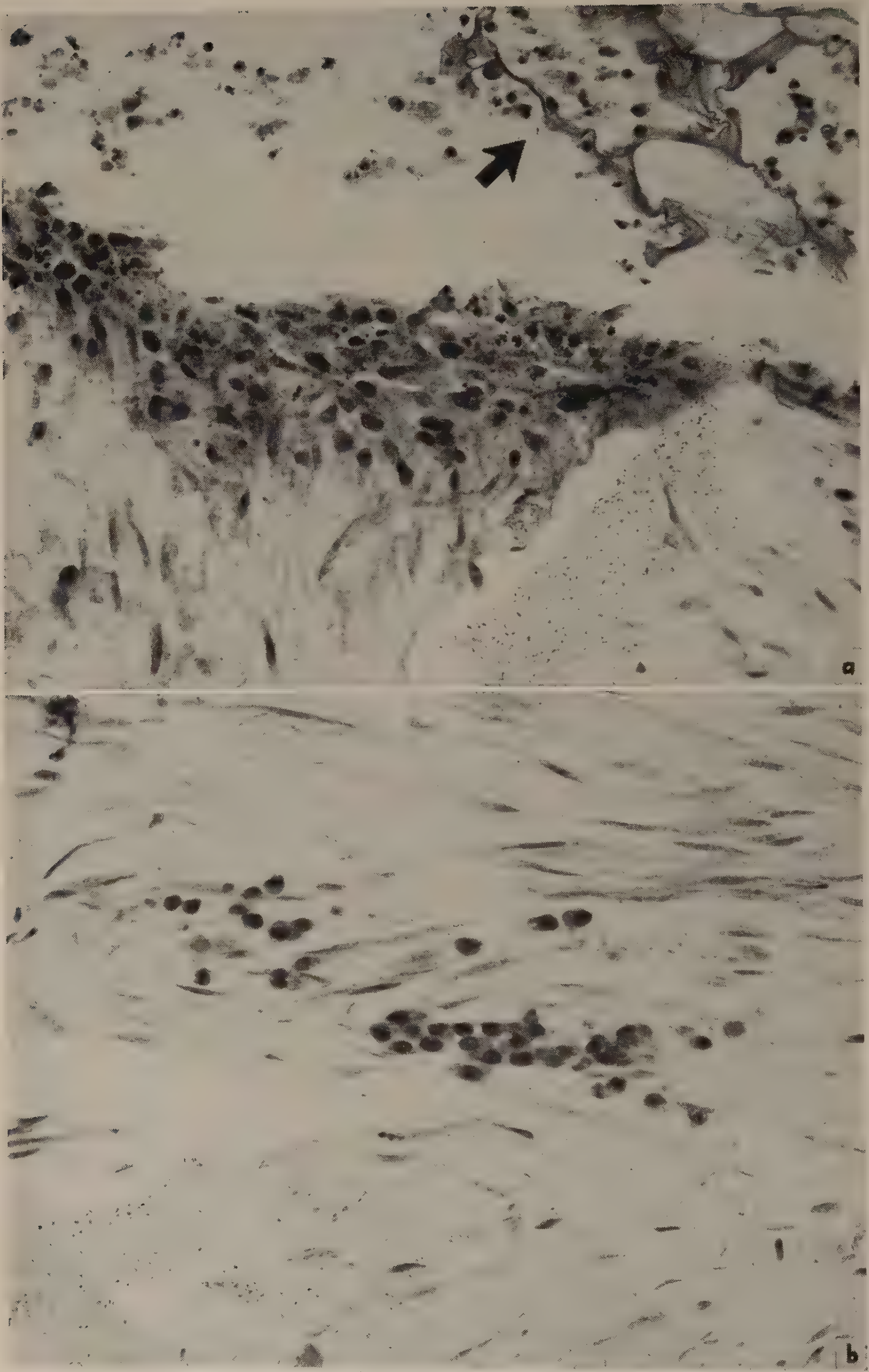

FIGURE 1. Comparison of the invasive spread of HeLa and D-189, observed when the lines are implanted in sponge-matrix cultures of normal human connective tissue. Hematoxylin and eosin. ×280. (a) HeLa and connective tissue. HeLa cells are seen extending from the inoculum, indicated by the Gelfoam (arrow) into the nearby normal tissue. (b) D-189 and connective tissue. Unlike HeLa, many D-189 cells are commonly found in the deep connective tissue at some distance from the inoculum. One of these distant sites is seen here, with the D-189 cells appearing singly and in short columns. At the site of inoculation, D-189 presents a picture of local infiltration similar to that of HeLa in 1a.

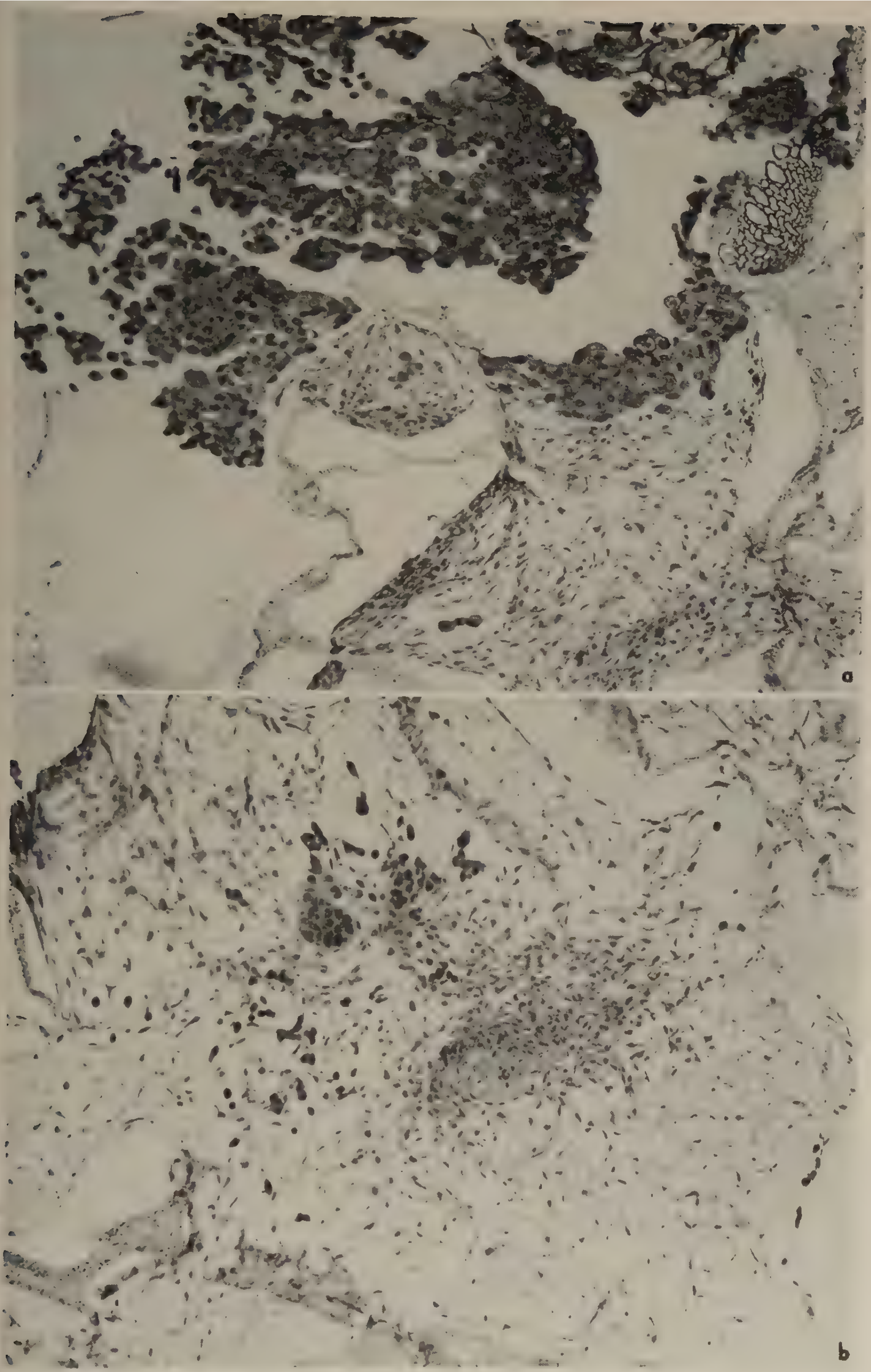

FIGURE 2. Comparison of the invasive spread of Conjunctiva and D 189 when each is added to sponge-matrix cultures of chick embryonic tissues. Periodic acid-Schiff. ×160. (a) Conjunctiva and chick tissue. Conjunctiva is seen on the surface of the host tissue near the Gelfoam explant. There is some infiltration of the normal tissue. (b) D-189 and chick tissue. Unlike Conjunctiva, D 189 cells are found in the depths of the sponge, sometimes in small clusters, as illustrated here. Near the inoculum, D-189 presents a picture of local invasion similar to Conjunctiva, as seen in 2a.

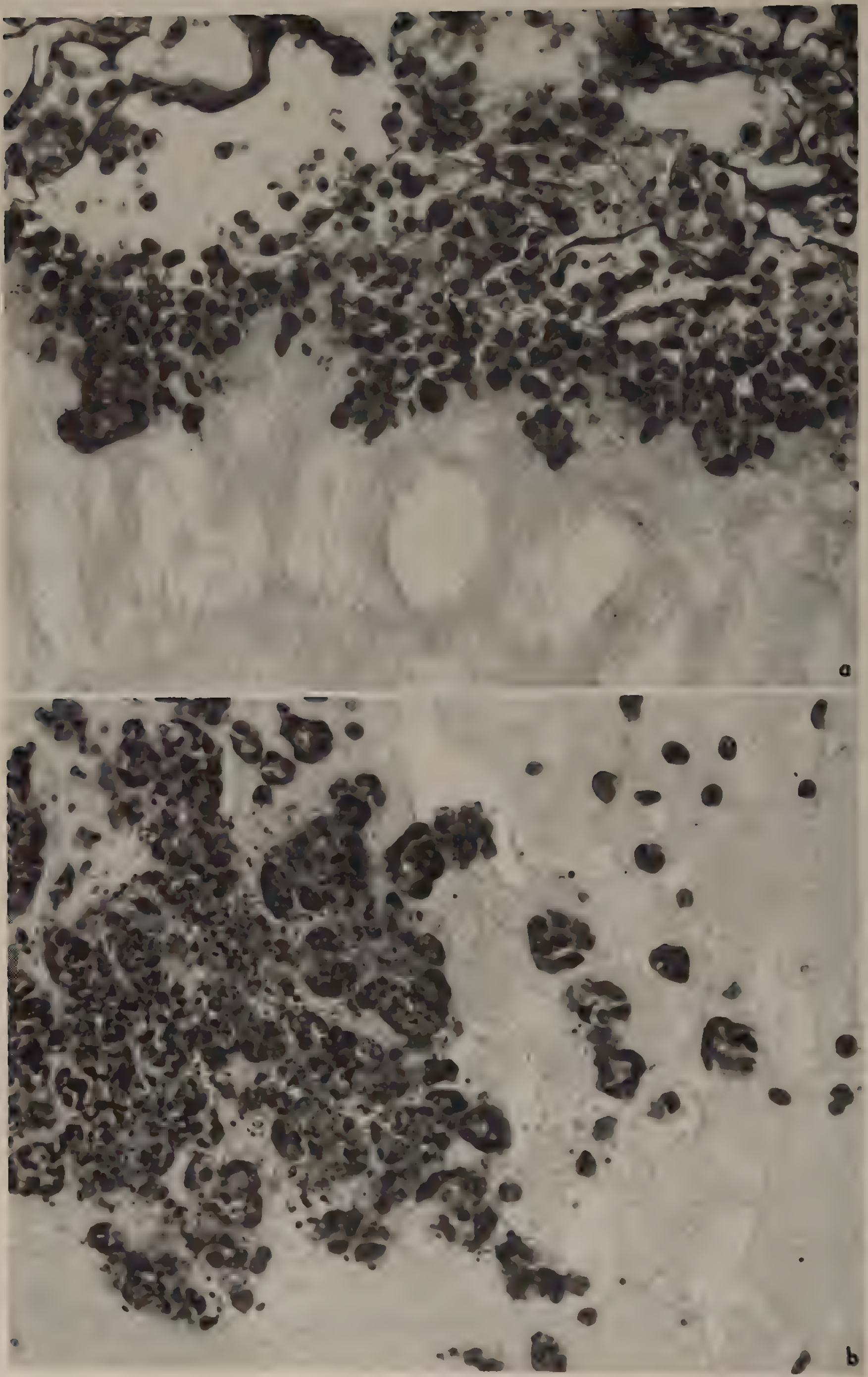

Figure 3. Comparison of the growth of Conjunctiva and D 189 in umbilical cord matrix. The difference between the two lines in this matrix is in the degree of spread from the Gelfoam inoculum. D 189 cells spread more extensively than do the Conjunctiva cells. Hematoxylin and eosin ×280. (a) Conjunctiva in umbilical cord. Conjunctiva cells are seen at the edge of the inoculum, infiltrating the contiguous substance of the cord. (b) D 189 in umbilical cord. A group of aggregates of D 189 is seen some distance from the site of Gelfoam implantation.

Agar-Overlay Technique

Earlier efforts at a quantitative expression of the degree of invasive spread in sponge-matrix tissue culture were frustrated by one property of the invading cells.[3] Clumps of cells tended to break off from the contiguous growth around the explant and were swept mechanically into the circulation of the roller tube to settle elsewhere and produce new centers of growth. To eliminate this factor, D-189 and Conjunctiva were compared in a system that minimized, and in some instances completely halted, the tendency of the fluid medium to scatter the cells mechanically. This was achieved by arranging the system so that the cells received all their nutrition by diffusion through an agar gel.

The hosts were luxuriant cultures of normal connective tissues of human fetal (MAF, TABLE 1) and chick embryonic origin, growing on coverslips. Minute inocula of D-189 or Conjunctiva growing in Gelfoam particle suspension were placed on the coverslips near or on the connective tissue growth and cemented in place with a thin plasma clot. The entire preparation was submerged beneath a 1-ml. volume of molten 1 per cent agar-medium mixture in a flat tube, and the mixture was allowed to gel. An overlay of 1 ml. of medium was added, and tubes were incubated at 35° to 37° C., usually in a stationary rack. The liquid nutrient was replenished from time to time as indicated by changes in *p*H.

A distinct difference was observed in the behavior of the two cell lines. Where Conjunctiva was implanted on a sheet of connective tissue, the outgrowth of conjunctival cells was mainly radial and contiguous. In some places distinct zones of demarcation were seen between the conjunctival and the normal connective tissue cells (FIGURE 4a). In the combinations with chick embryonic connective tissue, clot liquefaction sometimes led to the envelopment of the conjunctival inoculum in a roll of connective tissue. Outgrowth of conjunctival cells here was relatively meager. Instead, the cells apparently were confined by the connective tissue.

Strain D-189 presented a completely different picture. In all instances, many D-189 cells were seen thriving in numerous areas distant from the site of the inoculum (FIGURE 4b). Even where the Gelfoam explant was wrapped in a sheet of connective tissue, D-189 cells were seen streaming into many connective tissue areas.

Animal Inoculation

Many transplantation studies have been made in an effort to better understand the nature of "transformed" cells.[10-13] The question usually under consideration in these studies is whether a particular cell line is malignant. The results in different laboratories, as well as their interpretation, are often in conflict. The confusion arises in part, we suspect, because the question asked is too vague. Malignancy has many facets, and considering it as a unity is not meaningful.

We have been intrigued by the small but definite "takes" that Berman *et al.*[12] have obtained with inoculation of some of their cell lines onto the chick chorioallantoic membrane. Apart from the question of whether these lesions that

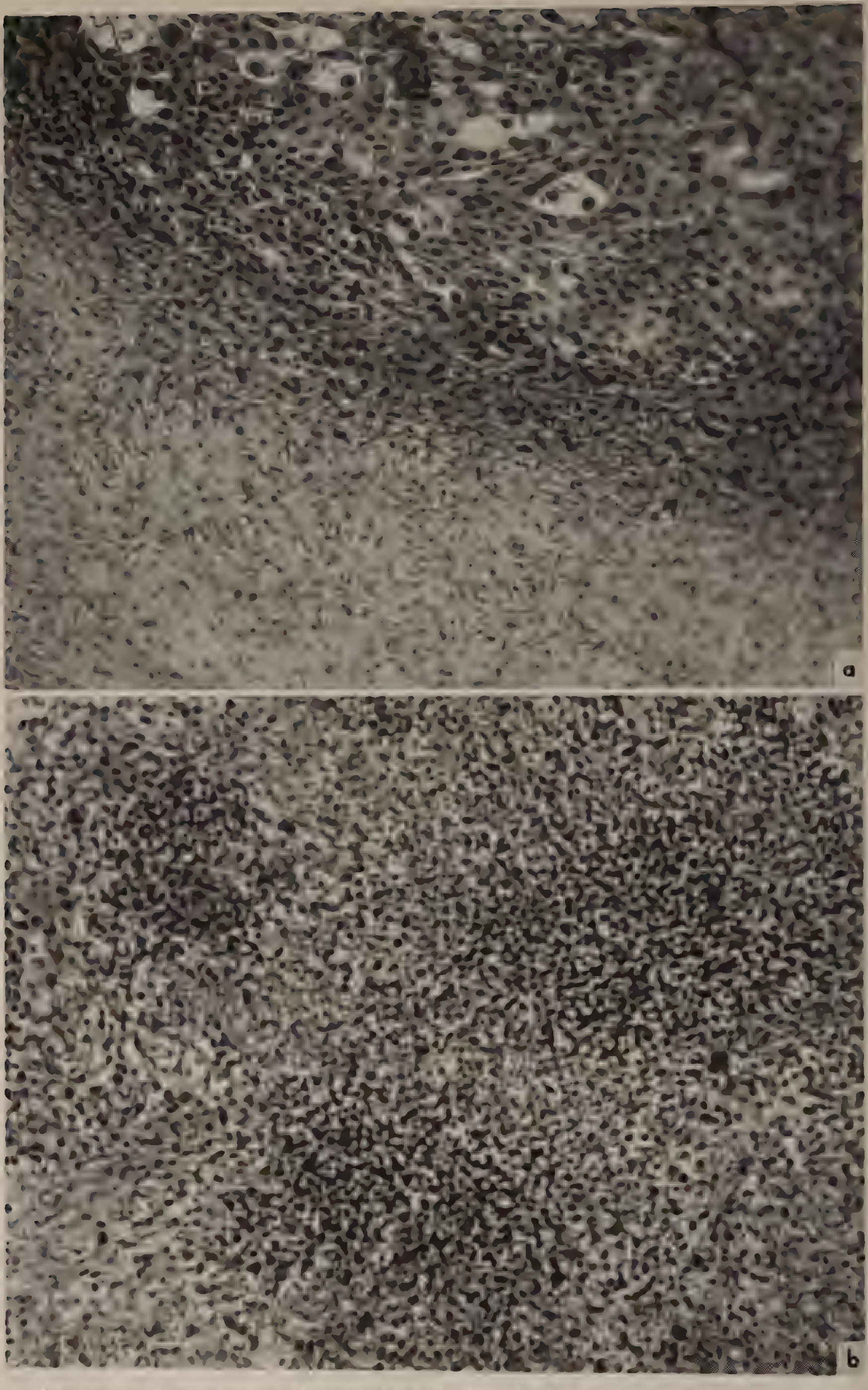

FIGURE 4. Comparison of the growth of Conjunctiva and D-189, each implanted on a sheet of chick embryonic connective tissue and then covered by an agar gel. Metabolites are exchanged by diffusion through the agar overlay. Hematoxylin and eosin. ×160. (a) Conjunctiva and connective tissue. Expanding, relatively contiguous growth of Conjunctiva cells is seen. A definite zone of contact between the Conjunctiva cells and the margin of the connective tissue can be identified. (b) D-189 and connective tissue. D-189 infiltrates and replaces the connective tissue cells diffusely, without the appearance of any boundary zone between the two kinds of cells. The pattern of infiltration is so diffuse that the normal and "transformed" cells are not readily identifiable.

look microscopically like cancer are or are not cancerous, such lesions, by their very size and relative histological simplicity, appear to lend themselves to a detailed morphologic analysis that is almost impossible with larger tumors.

Recently we have been studying cell lines on the chick chorioallantoic membrane in two ways. The first is direct inoculation onto the chorioallantoic membrane of 8- to 10-day-old embryonated eggs. Here the chick tissue alone is host. The second way is to inoculate the cell lines into pieces of freshly excised infant's foreskin, and then place the foreskin-cell line combination onto the chorioallantoic membrane of the egg (companion hosts). Here the hosts in direct contact with the "transformed" cells are both the surviving elements of the human skin and the components of the chick stroma that enter the skin.

Direct inoculation. We have compared the lesions produced by seven different cell lines inoculated directly onto the chorioallantoic membrane. The number of nodules examined up to this time is not great enough for us to know with certainty that any particular kind of lesion is specific for a particular cell line, although there are indications that such may be the case for some cell lines. The lesions vary considerably in many respects, such as compactness, size, the response of the overlying squamous epithelium, and the response of of the host connective tissue growing between the aggregates, within the conglomerate nodules, and around the nodules.

On casual examination all the lesions appear to be similar. The similarities are chiefly that the cells are large and hyperchromatic and occur characteristi-

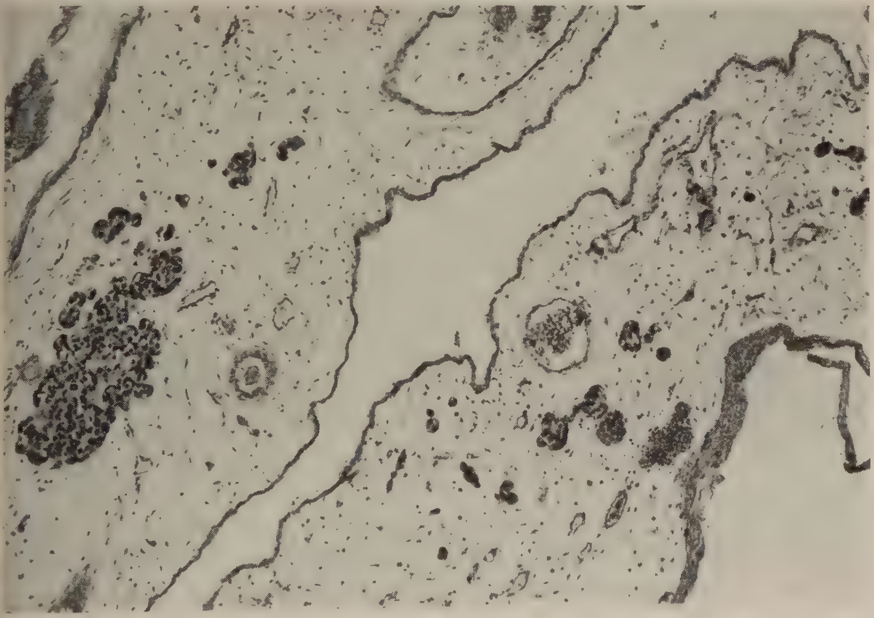

FIGURE 5. Nodules produced by D-189 implanted on the chorioallantoic membrane of the chick. Aggregates of cells touch one another to form small compact conglomerations. In addition, single aggregates are found at various places in the membrane. Hematoxylin and eosin. Approx. ×130.

cally in small groups or aggregates. One rarely sees an implanted cell completely free of contact with another of its own kind. Each aggregate of cells commonly appears on section as a short cord or acinar grouping. Conglomerations of these aggregates make up the tumor nodules. The arrangement of the aggregate building blocks within the nodules varies from one nodule to another. Some aggregates appear to be in intimate contact with each other. Others are separated by sparsely cellular, finely fibrillary, edematous connective tissue in which metachromasia often can be found. The arrangement of collagen fibers varies. In some instances there appears to be almost no increase of collagenous fibers around the tumor nodules; in others, thin laminae of collagenous fibers are frequently found. Collagen fibers may only encircle the tumor nodules, or may be arranged in an irregular, radial latticework extending into the tumor nodule between aggregates for a span of the diameter of several aggregates. Not all the cell lines grow primarily as conglomerate nodules; some appear to grow equally well as scattered aggregates in the mesodermal layer without apparent microscopic evidence of a response of the adjacent connective tissue to their presence.

Differences between cell lines appear to be expressed in terms of these qualities. HeLa and Conjunctiva are similar in that they form large nodules readily. The connective tissue between the HeLa aggregates frequently is

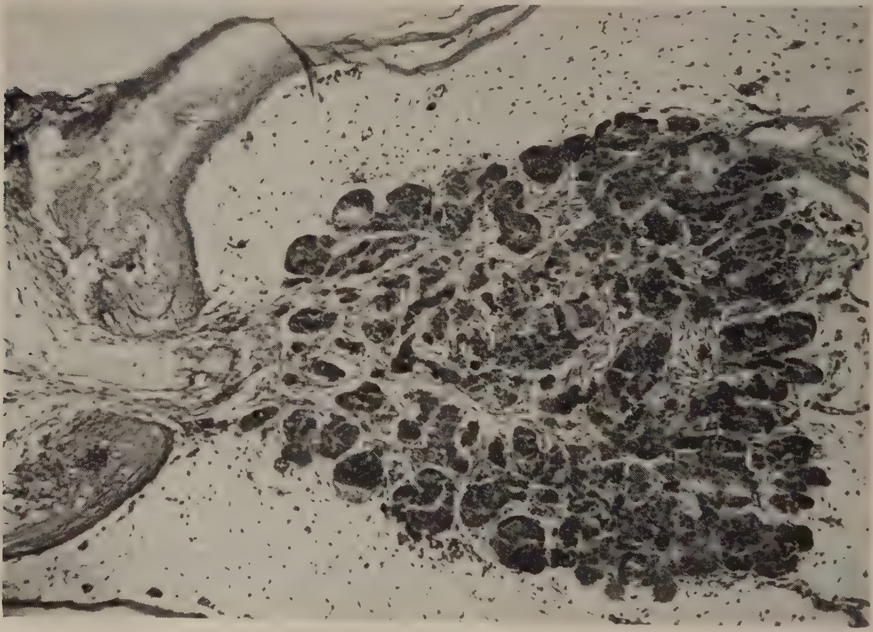

FIGURE 6. Nodule produced by Conjunctiva implanted on the chorioallantoic membrane of the chick. Hyperplastic squamous epithelium near the nodule shows a break in continuity through which granulation tissue protrudes. The aggregates are separated from one another by aisles of cellular, fibrillary connective tissue. Hematoxylin and eosin. Approx. ×130.

metachromatic. This is less common for Conjunctiva. D-189 grows as smaller conglomerations in which the acinar units usually are in direct contact with one another. Furthermore, scattered, isolated aggregates are seen in different areas of the connective tissue without evidence of local connective tissue response.

Some of these variations are illustrated in FIGURES 5, 6, 7, and 8. Different lesions appear to be "put together" in different ways.

Companion hosts. The modification of the direct implantation method in which both the human skin and the chick embryo serve as hosts for the "transformed" cells will be readily recognized as being fundamentally the same system used by Goodpasture *et al.*[14, 15] to study the growth of viruses. To date, only strain HeLa has been examined in this system.

In one experiment, 6 disks of foreskin were prepared with a biopsy instrument. Minute clumps of HeLa cells were implanted on the under side of the pieces of skin, that is, the connective tissue surface. These were then placed on the chorioallantoic membranes of 8-day-old embryos, with the tumor cells sandwiched between the human host and the embryonic host. Ten days later 5 embryos were alive. The skin implants in each case were well vascularized.

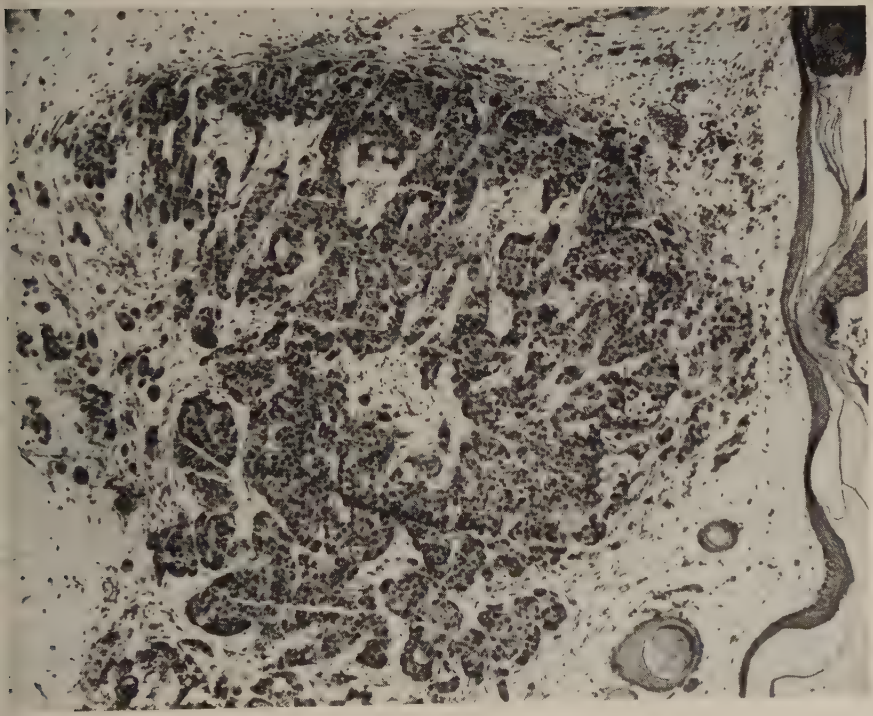

FIGURE 7. Nodule produced by strain HeLa implanted on the chorioallantoic membrane of the chick. The connective tissue between the aggregates is sparsely cellular and consistently metachromatic. Hematoxylin and eosin. Approx. ×130.

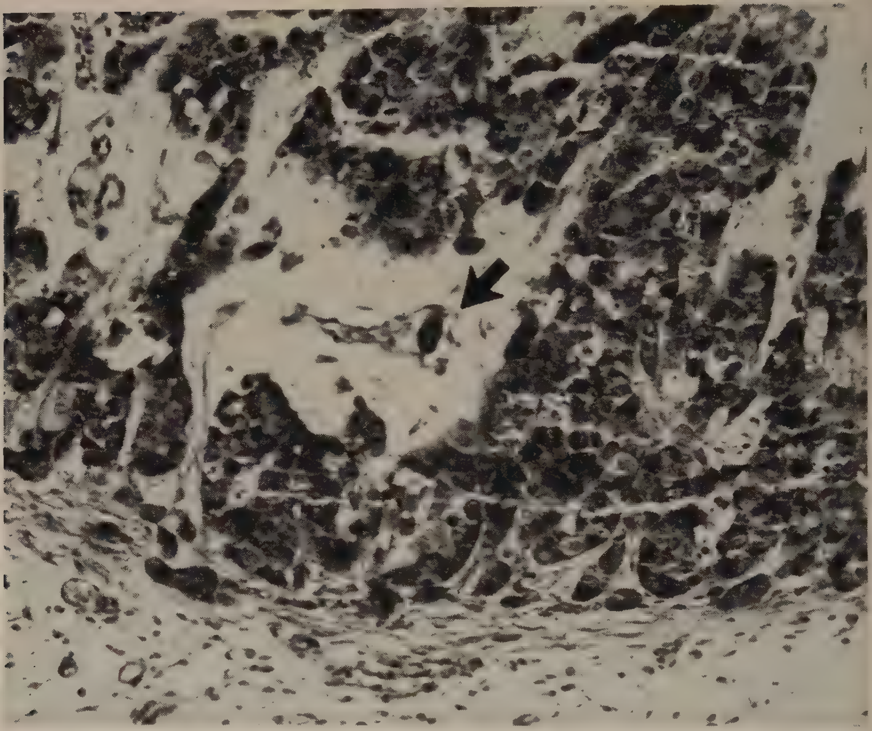

FIGURE 8. Detail of the HeLa nodule seen in FIGURE 7. HeLa cells are observed within the lumen of a small blood vessel (arrow). Periodic acid-Schiff. ×280.

In histological sections, HeLa cells were seen in 4 of the 5 specimens. Tumor cells were found in the zone of contact between human and chick tissue and infiltrating the connective tissues of each of the 2 host participants (FIGURE 9).

Discussion

Continuous lines of human cells are of interest in cancer chemotherapy as potential instruments for the discovery of anticancer agents. In this context, correlations have been sought between cytotoxicity *in vitro* and efficacy *in vivo*. Eagle and Foley[16, 17] have demonstrated that a remarkable parallel exists between the compounds that show antitumor activity in tumor-bearing animals and those that show toxicity for a variety of cell lines.

From the point of view of reasonable classification, the great number of cell lines developed in the past five years has produced a chaotic picture. We all recognize the need for meaningful classification of the cells, but the selection of the most important facts on which to base such organization presents many problems. Derivation from normal or from cancerous tissue does not appear to provide a suitable basis for the needs of cancer chemotherapy or for any purpose other than a historical one. Compared in terms of derivation, HeLa

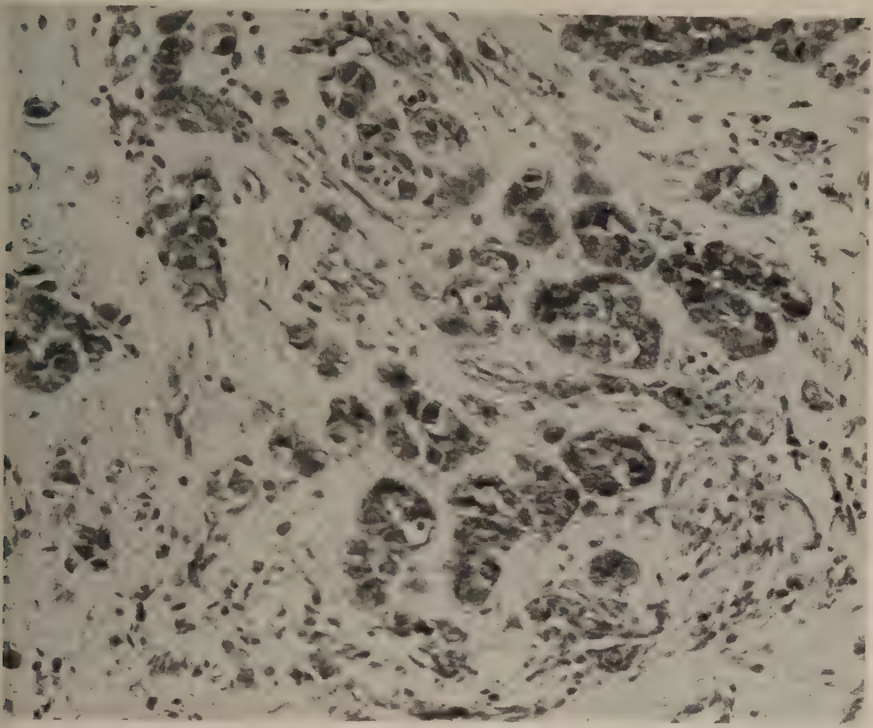

FIGURE 9. Infiltrative growth of HeLa cells into infant's foreskin. The cells were placed on the underside of freshly excised skin, and the combination was implanted on the chorioallantoic membrane of the chick. Invasive spread can be seen in the surviving connective tissue of the skin. Hematoxylin and eosin. ×280.

cells are found in one laboratory to be less malignant on heterologous transplantation than cells derived from normal tissue,[18] while in another laboratory they were found to be apparently more malignant.[11] If one considers the wide variety of selective factors that act on a line of cells from one laboratory to another, it is not surprising that, as time passes, the tissue of origin has less and less significance. Among the more obvious variations in handling are temperatures of incubation, composition of the medium, intervals between feedings, ratio of nutrient volume to cell population, and cell crowding.

Classification for the needs of cancer chemotherapy may not be the same to a cytologist, geneticist, or biochemist. One is reminded of such problems in bacteriology, where organisms are classified as Gram-positive or Gram-negative, motile or nonmotile, aerobes or anaerobes, pathogens or nonpathogens. Working classifications for the cancer biologist probably will be concerned finally with the varied qualities of pathogenicity or nonpathogenicity for the host bearing the cancer. It may be necessary to use other more easily accessible differences temporarily as stepping stones to this goal.

Studies on elements of pathogenicity are possible today, however. We have presented some observations on several cell lines that probably relate directly to this topic. Direct differences have been found between two cell lines when they are compared in several tissue culture systems involving normal tissues as hosts. A comparison of several cell lines inoculated onto the chorioallantoic membrane of the chick suggests that differences between cell lines also may be recognized by a careful scrutiny of the tumors they produce, including the responses of the host to the tumor. Lesions produced by different cell lines vary in their general architecture in terms of their compactness and the occurrence and spatial distribution of collagen fibers, capillaries, and metachromasia.

In recent experiments we have placed HeLa cells in a host environment that includes features of homologous and heterologous transplantation. Many years ago Goodpasture observed that pieces of skin transplanted to the chorioallantoic membrane of the chick retained their human qualities to the extent that viruses could be cultivated on them and could produce characteristic lesions. We are using such transplants for the bioassay of the pathogenicity of the cell lines and are exploring the variations of *in vitro* cultivation that will alter malignancy as appraised in this system.

We hope that efforts at evaluating cell lines in terms of their pathogenicity eventually will clarify many of the components of the complex phenomena of malignancy. Perhaps the goal of effective treatment of cancer will be reached by elucidation of the mechanisms that control host-tumor interactions in local invasiveness as well as in distant implantation, which is also a local phenomenon.

Summary

We have been using continuous lines of human cells to study the mechanisms of tumor invasion with sponge-matrix tissue culture and related techniques. The first of the continuous lines of human cells (HeLa), when combined with normal connective tissue in sponge matrix, was seen to invade and replace the normal tissue. Other lines of cells derived from cancer (KB and J-96) showed a similar picture of invasion in the same system.

As newer cell lines emerged, including some from normal tissue, a comparison of the invasiveness of "cancer" and "normal" cell lines was possible. Morphologic examination of several continuous lines from normal tissues indicated that they were almost indistinguishable from the lines obtained from cancer tissue. It was hoped, however, that distinctive qualities would appear when "cancer" and "normal" lines were combined in sponge matrix with recently isolated normal connective tissue. Several lines have been surveyed in this system. Our greatest experience has been in comparisons of Chang's Conjunctiva and a histologically malignant derivative of normal infant's foreskin (Strain D-189). These two cell lines differ markedly in their capacity to invade normal connective tissue *in vitro*.

Strain D-189 is highly invasive for connective tissue; cells of this strain can be found at great distances from the inoculum, deep in the connective tissue, a few days after inoculation. Chang's Conjunctiva is locally invasive and does not spread widely. In some cultures there is a suggestion that fibroblasts wall

off the growth of the conjunctival cells. Our stock strain of HeLa cells appears to fall between the two lines of normal origin in its invasiveness. When examined with our system, the extremes of high and low invasiveness are observed between two cell lines, both of which were derived from normal tissue.

Recently we have examined several lines of human cells *in vivo* by planting them on the chorioallantoic membrane of the chick embryo. Differences have been seen in the architecture of the small tumors that develop from different lines. Of particular interest is the variation in response of the normal connective tissue of the chorioallantoic membrane to the presence of the tumor. The lesions produced by different lines appear to be "put together" differently in terms of their compactness, location, and the distribution of collagenous fibers, as well as in the metachromasia of the ground substance. The lesions can be readily overlooked on casual gross inspection. They lend themselves, however, to histological study; complete serial sections may be examined easily, and the architecture of the tumor and its spatial relationship to the host tissue clearly recognized.

Currently we are examining the interaction of cell lines and normal human skin, transplanted together on the chick chorioallantoic membrane. The skin serves as a homologous tissue and the chick as a heterologous host. HeLa cells have been found to invade both the human skin and the chorioallantoic membrane. Other lines of cells are being assayed as well.

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Discussion of the Paper

Harry Eagle (*National Institute of Allergy and Infectious Diseases, Public Health Service, Bethesda, Md.*): We are faced with a paradox. In Foley's system there is a remarkably uniform and quantitatively striking difference in the behavior of these serially propagated strains on inoculation into the cheek pouch of the normal hamster. These are not even remotely as irregular in the case of Moore's system. In Leighton's system some of his "invasive" lines are derived originally from normal tissue. Does either Moore or Foley have any data on D-189 which, for Leighton, is highly invasive with respect to implantation?

Alice Moore (*Sloan-Kettering Institute for Cancer Gesearch, New York, N. Y.*): No. I do not.

G. E. Foley (*The Children's Cancer Research Foundation, Boston, Mass.*): The strain of this cell line that we have handled came from Microbiological Associates, Inc., Bethesda, Md., and it behaves as a normal cell in hamsters. It does not grow from inocula containing less than 100,000 cells.

Eagle: This certainly points up what Leighton said, that we find any answer we want, depending upon the criterion we choose.

Leighton: Part of the resolution of this conflict comes from clues that H. S. N. Greene gave us several years ago. Greene planted highly anaplastic tumors into the anterior chamber of the eye of the guinea pig. In some instances tumors that failed to grow alone in this site did so when a fragment of fetal lung was inoculated into the eye at the same time. The fetal lung appeared to provide some sort of stroma.

In vitro and *in vivo* systems are radically different, so one should not try to apply *in vitro* data directly to an *in vivo* system. I think that D-189 and HeLa can be better understood if we combine Foley's experience and mine and keep in mind Greene's observations. HeLa in the chick apparently evokes a striking stromal response. This, I suspect, is very important for the survival of the HeLa cells. D-189, although invading widely *in vitro*, does not evoke a stromal response. Its growth *in vivo* appears to resemble that of a tissue culture. Probably, when planted in the cheek pouch of a hamster, cells such as D-189 fail to evoke very much of a connective tissue response. They cannot grow as a compact "tissue culture" *in vivo* very long either, for in this condition they would become ischemic and die. Cells such as HeLa that can evoke a suitable stromal bed do survive and produce a tumor.

Eagle: I am still puzzled. After all, why will it not grow—eliciting no stromal response *in vivo* when it grows on a relatively simple medium *in vitro*? Everything in the *in vitro* medium on which it will grow is present in the tissue fluids.

Leighton: D-189 evokes a meager stromal response. Perhaps its survival *in vivo* in such hosts as the chorioallantoic membrane is attained through the ability of small groups of cells to separate from the growing mass at the site of inoculation and thus obtain adequate nutrition from the normal connective tissue by diffusion. If small groups of cells or individual cells were unable to migrate from the nodule at the site of implantation, continued growth of the implant as a compact mass would lead to the death of the implant. HeLa apparently survives by inducing changes in the stroma that bring adequate nutrition to the tumor. D-189, lacking this mechanism, appears to wander out into the normal connective tissue for its nutritional needs.

TUMOR-SPECIFIC CYTOTOXICITY OF ANTITUMOR AGENTS*

By John J. Biesele

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The title chosen for this paper represents an ideal rather than a reflection of the activity of anything presently available in the armamentarium of cancer chemotherapy. I propose to consider some reported occurrences of selectively toxic effects of agents on certain neoplastic cells in culture and the possibilities of applying such differential effects to a screening program.

Four years ago I discussed tissue-culture assay of antitumor agents in a previous publication of The New York Academy of Sciences (Biesele, 1954). Limitations of my procedures, which involved various morphologic examinations of the effects of agents on short-term cultures of freshly explanted tissue in classic media, and of the results were pointed out. For several agents, notably 6-mercaptopurine, "pyridoxine-nitrogen mustard," and some antifolic acids, gross damage selective to cells of mouse Sarcoma 180 in culture was not related to effectiveness of the agents against this sarcoma *in vivo*, but a differential suppression of mitosis in the sarcoma cells, relative to embryonic mouse skin fibroblasts in culture, appeared to be so related. For such agents as a group of ethylenimines, no differential effects in gross damage or in inhibition of mitosis were noted in culture, but the toxicity to either neoplastic or embryonic cells in culture was correlated with activity against Sarcoma 180 in the mouse.

It therefore appeared desirable to stress caution in the interpretation of tissue-culture assays and to avoid extending application of the results beyond the particular combination of tumor, normal tissue, and culture conditions employed, so long as we remain unsure of the existence of a hypothetical unique difference susceptible to chemical attack between all neoplastic cells on the one hand and all normal cells on the other.

Since then we have looked in more detail into the differential inhibition of mitosis in sarcoma cells relative to embryonic skin fibroblasts. Our conclusion is that this differential inhibition of mitosis in culture is not a completely reliable indicator of what will happen when the same agent is applied to the same tumor in an animal. This conclusion springs from a comparison of related agents and from comparisons of the effects of a given agent under variously changed conditions.

For the first case we note that, on 24-hour exposure, 6-mercaptopurine inhibits Sarcoma-180 mitosis rather more than that of embryonic skin fibroblasts in culture, but thioguanine inhibits mitosis in these cells to about equal extents (Biesele, 1958b). Although azaserine or gamma-glutamyl hydrazide inhibit mitosis more in Sarcoma 180 cells than in fibroblasts, the reverse is true of DON over most of the tested range (Biesele, 1958a).

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For comparisons of effects under different conditions, consider 6-mercaptopurine. Addition of insulin or lipoic acid (Biesele, 1958b) or cysteine or cystine (Biesele, 1957) to cultures treated with 6-mercaptopurine tends to increase the mitotic inhibition of embryonic fibroblasts to such an extent that one no longer speaks of a selective inhibition of Sarcoma 180 mitosis. There is a somewhat similar tendency expressed in chromosome breakage (Biesele, 1958a). One can choose a concentration of coenzyme A that will largely antagonize the considerable mitotic inhibition of Sarcoma 180 cells caused by 1.0 millimolar 6-mercaptopurine, but will have little influence on the moderate inhibition of fibroblasts brought about by 6-mercaptopurine (Biesele, 1955).

Experiences such as these leave me with little doubt that cells of different types and origins in tissue cultures sometimes can be brought to react differently to an agent. At the same time, these differences are often not great, and they may be readily subject to environmental manipulation. The medium may play a large role in cellular responses, especially to antimetabolites. We must remember that, from the standpoint of the medium for mammalian cells, our departure is never from an uncomplicated base.

Eagle and Foley (1956) have published intriguing reports of activity of certain agents at extremely low concentrations in tissue culture. Their experimental procedure differs from ours, notably in refeedings and redosings carried out about five times over the course of a week. Usually we have not refed, but have dosed two days after planting and have examined after 24 hours' exposure.

Accordingly, we carried out several experiments with modified procedures. Refeeding at the time of the single dosing has little apparent effect, as seen in TABLE 1, with unsubstituted purine.

However, multiple dosing produces a decided magnification of effect. TABLE 2 illustrates this for 6-mercaptopurine at two dosings. With 1.0 millimolar 6-mercaptopurine, the majority of the sarcoma cells had died after two dosings, and the fibroblasts had increased the extent of their inhibition to the level reached by the sarcoma cells after a single dose. Five dosings, it may be imagined, might increase inhibitions on a great scale.

In conclusion, let me emphasize my lack of knowledge of an agent with absolute tumor-specific cytotoxicity. There appear to be agents that under certain conditions will damage cells of one sort more than those of another sort, but doses damaging to both may be separated by only small concentration steps. Eagle and Foley (1956) give instances of agents whose 50 per cent inhibitory doses for different cell strains differ by as much as twofold to tenfold, but they consider these differences in general to lack significance or to be anomalous. The fact that one can modify the extent of mitotic inhibition caused by some antimetabolites, including antitumor agents, with relative ease also diminishes the significance of differential inhibitions for screening purposes. However, if this narrow and foggy field of differential inhibitions is the one in which the cancer problem is to be engaged, I suggest that we enlarge our knowledge of the field, as well as look to our chemotherapeutic weapons with suitable screens.

TABLE 1

LACK OF INFLUENCE OF REFEEDING AT TIME OF DOSING ON INCIDENCE OF MITOSIS AND OF NUCLEAR DEGENERATION IN MOUSE TISSUE CULTURES AFTER 24-HOUR EXPOSURE TO UNSUBSTITUTED PURINE HYDROCHLORIDE

	Sarcoma 180		Skin fibroblasts	
	Mitotic	Degenerate	Mitotic	Degenerate
	%	%	%	%
Not refed				
Controls	15	1	8	1
Purine, 0.1 μM/ml	0	79	9	1
Purine, 0.01 μM/ml	9	2	7	1
Refed with serum & embryo extract				
Controls	13	1	9	1
Purine, 0.1 μM/ml	0	87	12	1
Purine, 0.01 μM/ml	7	9	15	1
Refed with Eagle's medium				
Controls	8	2	14	1
Purine, 0.1 μM/ml	0	35	12	2
Purine, 0.01 μM/ml	7	2	12	1

TABLE 2

INFLUENCE OF REPEATED DOSING WITH 6-MERCAPTOPURINE ON INCIDENCE OF MITOSIS AND OF NUCLEAR DEGENERATION IN MOUSE TISSUE CULTURES

	Sarcoma 180		Skin fibroblasts	
	Mitotic	Degenerate	Mitotic	Degenerate
	%	%	%	%
Fed & dosed day 3, fixed day 4				
Controls	13	0	10	1
6-Mercaptopurine, 1.0 μM/ml	2	7	6	1
6-Mercaptopurine, 0.25 μM/ml	3	6	8	1
6-Mercaptopurine, 0.0625 μM/ml	10	0	10	1
Fed & dosed days 3 & 5, fixed day 6				
Controls	8	2	8	2
6-Mercaptopurine, 1.0 μM/ml	0	56	2	10
6-Mercaptopurine, 0.25 μM/ml	1	5	9	2
6-Mercaptopurine, 0.0625 μM/ml	7	2	9	2

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SUSCEPTIBILITY OF CULTURED HUMAN CELLS TO ANTITUMOR AGENTS*

By Harry Eagle

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Introduction

In a previous study[1] a limited number of agents known to have an antitumor effect in animals or man were found to be highly cytotoxic against several human and animal cells in tissue cell cultures, while several compounds devoid of antitumor activity failed to exhibit significant cytotoxicity against these same cells *in vitro*. This suggested that cytotoxicity in cell cultures might be a useful primary screen for the detection of potential antitumor agents. The present studies were undertaken to obtain additional information on this point.

Materials and Methods

A series of 200 compounds taken from the files of the Cancer Chemotherapy National Service Center (CCNSC), Bethesda, Md., was used in these studies. These 200 compounds were not chosen at random, but were selected to include known antitumor compounds, as well as agents highly active in other biological systems but not known to possess antitumor activity. The evaluation of the activity of these compounds in experimental tumor systems was based on information provided by the CCNSC and supplementary mouse tumor tests,[2] and has been described in detail in a previous paper in this monograph[3] and elsewhere.[4] The identification of the compounds and their individual cytotoxic activity, together with the available data on experimental antitumor activitiy, also have been detailed in this monograph[3] and elsewhere.[4]

The present analysis will consider only those compounds in the series that had been tested in a least 3 experimental tumor systems. The compounds were classified as "tumor-active, ++" if 2 or more tumor systems were affected, and "tumor-active, +" if only 1 experimental tumor was affected. A compound reported to be positive in 1 tumor system and with borderline activity in another was classified arbitrarily as "tumor-active, ++," while borderline activity in 2 experimental tumor systems was adjudged as "tumor-active, +."

Obviously there are many aspects of animal tumor assays that limit their reliability as a frame of reference by which to evaluate other screening procedures.[3, 4] This reference base has been used in the present analysis only because of the present lack of a better one.

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One compound in the series could not be tested because of insolubility; 1 compound was duplicated; and, at the time of analysis, 18 compounds had not been studied in 3 experimental tumor systems. These 20 compounds were omitted from consideration.

Two human cell lines were used in these studies, one isolated from normal adult human liver[5] and another, Strain KB, derived from a human nasopharyngeal epidermoid carcinoma.[6] Each compound was titrated in duplicate serial tenfold dilutions against standardized inocula of each cell line. The technique has been described in detail elsewhere.[1, 4] The fluid in each culture was replaced after 2, 4, 5, and 6 days' stationary incubation at 37° C., and the extent of cell growth was measured by determination of the protein content of the flask. The inhibitory effects of the compound were expressed as the concentration range (1.0×10^{-3} to 1.0×10^{-4} gm./ml., 1.0×10^{-4} to 1.0×10^{-5} gm./ml., etc.), within which there was 50 per cent inhibition of growth (ID_{50}). A compound was classified arbitrarily as "positive" if the ID_{50} against both cell lines was 1.0×10^{-4} gm./ml. or less.

Comparison of results obtained with the two cell lines. Although each of the two laboratories used a different cell line, the results obtained with a given compound usually were in agreement; that is, inactive or "negative" compounds were so classified by both cell lines, and the active or positive compounds were cytotoxic in approximately the same concentration range. When the degree of cytotoxicity exhibited by a "positive" compound differed with the two cell lines, the higher value was used. It is of interest that, in most of these instances, the more sensitive cell line was that derived from normal tissue.

Eleven compounds were cytotoxic at 1.0×10^{-4} to 1.0×10^{-5} gm./ml. for only 1 cell line. These compounds were classified arbitrarily as negative in cell cultures. Two compounds were cytotoxic at 1.0×10^{-5} to 1.0×10^{-6} gm./ml. for 1 cell line, but inactive at 1.0×10^{-4} gm./ml. for the other. These 2 compounds were arbitrarily classified as positive.

Results

Cytotoxicity of compounds reported to be tumor active. In this series of 180 compounds, 89 agents were reported to exhibit antitumor activity in experimental tumor systems.[2] Of these, 68 were classified as tumor-active, ++, and 21 were classified as tumor-active, +, having been reported to exhibit activity in 2 or more and only 1 experimental tumor systems, respectively.[3, 4]

The cytotoxic activity of the tumor-active, ++, tumor-active, +, and tumor-negative compounds is summarized in FIGURE 1. The differences in the cytotoxicity of the tumor-active and tumor-negative compounds are immediately apparent. For example, 46 per cent of the 89 tumor-active compounds were cytotoxic at 1:1,000,000 or less, 72 per cent at 1:100,000, and 79 per cent at concentrations of 1:10,000 while, of the 91 tumor-negative compounds, the corresponding percentages were 2, 10, and 21, respectively.

Cytotoxicity of compounds reported to be tumor negative. As judged by the results in animal tumor systems, 19 (21 per cent) of the 91 compounds in the series reported to exhibit no antitumor activity in at least 3 *in vivo* systems

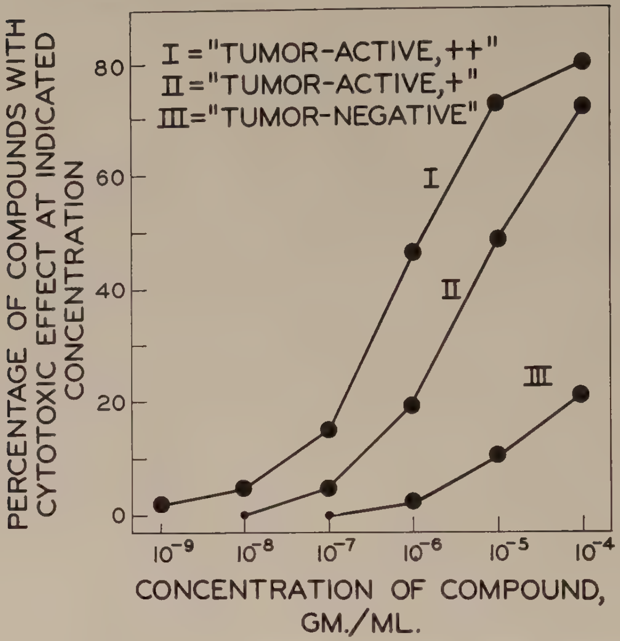

FIGURE 1. Cytotoxicity of 180 reference compounds in human cell cultures.[4]

gave "false-positive" results in cell cultures. As pointed out previously,[3, 4] since the compounds tested did not represent a random series, these data do not necessarily reflect the frequency of false positives that might be expected in a truly random group of candidate compounds, since many of the agents included in the present studies were known to be highly active in various other biological systems.

Even within this unrepresentative series, however, the tumor-negative compounds were significantly less cytotoxic than the agents active against two or more experimental tumors (FIGURE 1). Of the tumor-negative compounds, 72 (79 per cent) were not cytotoxic in cell culture at 10^{-4} gm./ml., as compared with the 14 (21 per cent) of the tumor-active, ++ compounds. Of the 19 so-called false-positive compounds, only 2 (2.2 per cent) were inhibitory at concentrations of 1.0×10^{-6} gm./ml., and none were inhibitory at 1.0×10^{-7} gm./ml.

Discussion

Response of "normal" and "malignant" cells in culture to cytotoxic agents. The present results with cell lines deriving from normal and malignant tissue substantiate the observation[4] that the majority of agents thus far known to exhibit antitumor activity are highly cytotoxic in cell cultures, and equally so against cell lines derived from normal and tumor tissues. It might be argued that cell cultures derived from normal tissue are no longer normal, but have acquired some of the characteristics of malignant cells, including sensitivity to antitumor agents. However, cell lines originally deriving from normal and malignant sources differ materially in their ability to grow and produce tumors in the cheek pouch of the golden hamster (*Mesocricetus auratus*), even after years of *in vitro* cultivation.[4, 7, 8] Furthermore, cells isolated *in vitro* from normal human and animal tissue and tested in their first tissue culture passage, before significant cell multiplication had occurred, were just as sensitive to antitumor agents as cells of normal (or malignant) origin that had been serially propagated in culture for years.[9]

It must be concluded, therefore, that most of the antitumor agents now available are highly and unselectively cytotoxic against normal and malignant cells *in vitro*. The selective antitumor activity exhibited by many of these compounds *in vivo* must be due to host-imposed differences that do not obtain under the culture conditions used in these studies.

Sensitivity and reliability of cell culture as a screening method. The results reported herein lend further support to the possibility that cytotoxicity in

TABLE 1

COMPOUNDS* REPORTED TO BE TUMOR ACTIVE THAT WERE NOT SIGNIFICANTLY CYTOTOXIC IN TISSUE CELL CULTURES

CCNSC X No.	Chemical name	Cytotoxicity in cell culture ID_{50} gm./ml.† versus:		Reported antitumor activity *in vivo*‡
		"Liver"	KB	
18	Isoriboflavin	10^{-4}–10^{-5}	$>10^{-4}$	2/4 (1±)
39	Galactoflavin	10^{-4}–10^{-5}	$>10^{-4}$	2/3
68	"AA-223"	10^{-4}–10^{-5}	$>10^{-4}$	3/4
143	4-Amino-6-anilino-1,2-dihydro-2,2-dimethyl-2-triazine	10^{-4}–10^{-5}	$>10^{-4}$	3/8 (2±)
158	Hydrocortisone	$>10^{-5}$§	$>10^{-5}$§	8/15
113	Isopropyl *N*-phenylcarbamate	$>10^{-5}$	$>10^{-5}$	2/6
20	Formamide	$>10^{-4}$	$>10^{-4}$	3/4
41	Ethyl carbamate	$>10^{-4}$	$>10^{-4}$	21/29
44	*N*-Methylacetamide	$>10^{-4}$	$>10^{-4}$	6/16
114	DL-Ethionine	$>10^{-4}$	$>10^{-4}$	5/22
134	2-Acetamido-1,3,4-thiadiazole	$>10^{-4}$	$>10^{-4}$	2/5
149	1,4-Dimethanesulfonyloxybutane (Myleran)	$>10^{-4}$	$>10^{-4}$	8/23
188	*N*-Methylformamide	$>10^{-4}$	$>10^{-4}$	11/17
197	2-Ethylamino-1,3,4-thiadiazole hydrochloride	$>10^{-4}$	$>10^{-4}$	3/6
21	DL-Desthiobiotin*	10^{-4}	10^{-4}	1/4
95	Folic acid*	10^{-4}	10^{-4}	2±/14
150	Resorcinol*	10^{-4}	10^{-4}	1/3
151	β-2-Thienylalanine*	10^{-4}	10^{-4}	1/19
186	5,6-Diaminouracil hemisulfate*	10^{-4}	10^{-4}	1/6
198	Benzotriazole*	10^{-4}	10^{-4}	1,2 ±/6

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* Compounds giving doubtful results in one or two tumor systems were included arbitrarily in this group.

† Concentration that caused 50 per cent inhibition of growth.

‡ Proportion of tumor systems inhibited.[2]

§ Could not be tested at higher concentration because of insolubility.

human cell cultures may be useful for primary detection of potential antitumor agents. Any screening device has two parameters: *sensitivity*, evidenced by the proportion of active compounds detected; and *reliability*, evidenced by the number of false-positive reactors. The parameter of reliability is of lesser importance in a screening device designed to detect the maximum number of positive agents by the simplest possible procedure provided, of course, that the frequency of false positives is not so high as to invalidate the usefulness of the screening method.

The evaluation of any screening procedure for antitumor agents is complicated by the lack of a reliable basis of comparison. The ultimate criterion is, of course, activity in human neoplasia. Since this cannot be used for the initial evaluation, it becomes necessary to compare one clearly fallible procedure (for example, activity *in vitro* against cell cultures or microorganisms) with another screening procedure (activity against experimental tumors) which may be equally fallible. With conflicting results, one does not know whether it is the positive result *in vitro* or the negative result *in vivo* (or vice versa) which is false in terms of activity against human cancer.[3, 4]

The obvious limitations inherent in any *in vitro* screen for the detection of potential antitumor agents also have been discussed previously.[3, 4] Considering these limitations, cell cultures were surprisingly effective in detecting the tumor-active compounds in the present series. Of the compounds with reported activity against experimental tumors, 79 per cent were cytotoxic at 1.0×10^{-4} gm./ml., 72 per cent at 1.0×10^{-5} gm./ml., 46 per cent at 1.0×10^{-6} gm./ml., and 15 per cent at 1.0×10^{-7} gm./ml. The corresponding percentages for the tumor-negative compounds were: 21, 10, 2, and 0, respectively.

It should be emphasized that all but two of these "cell culture positive"

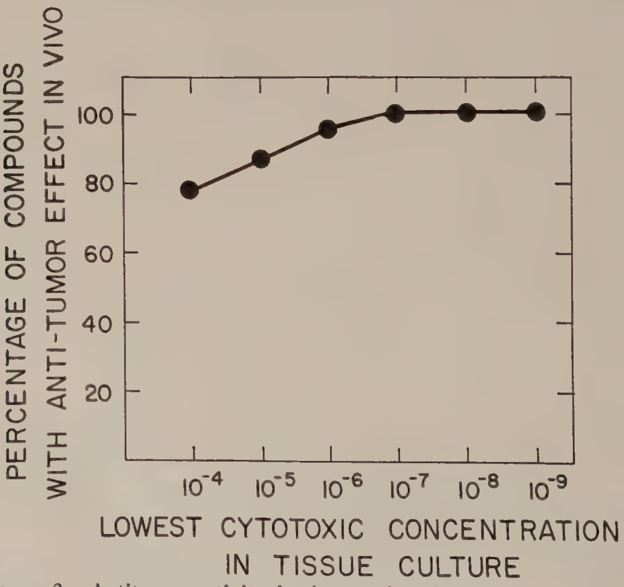

FIGURE 2. Antitumor activity *in vivo* as a function of cytoxicity *in vitro*.

compounds were so classified by a single, relatively simple test system. On the other hand, the experimental tumor data[2] represent results requiring the use of a wide variety of experimental tumor systems. It should be noted also that any one of these tumor systems alone would have classified as positive only a fraction of these tumor-active agents.

The tumor-active compounds missed by the cell culture screen are listed in

TABLE 2

COMPOUNDS REPORTED TO BE TUMOR NEGATIVE THAT WERE SIGNIFICANTLY CYTOTOXIC IN HUMAN CELL CULTURES

CCNSC X No.	Chemical name	Cytotoxicity in cell culture ID_{50} gm./ml.*	Reported antitumor activity *in vivo*† [2]
6	6-Methoxyquinoline-4-aldehyde	$>10^{-5}$ $<10^{-4}$	0/4
77	2,6-Dimethyl-4-(2-oxo-2-phenylethylmercapto)-pyrylium bromide	$>10^{-5}$ $<10^{-4}$	0/3
89	Chloramphenicol (Chloromycetin)	$>10^{-5}$ $<10^{-4}$	0/20
94	2,4′-Sulfonyldiphenol	$>10^{-5}$ $<10^{-4}$	0/3
100	Methyl chloroacetate	$>10^{-5}$ $<10^{-4}$	0/3
111	8-Azaxanthine	$>10^{-5}$ $<10^{-4}$	0/23
121	Ethylhydrocupreine hydrochloride	$>10^{-5}$ $<10^{-4}$	0/3
126	4-Amino-6-(*p*-chloroanilino)-1,2-dihydro-2,2-dimethyl-*s*-triazine·1 H_2O	$>10^{-5}$ $<10^{-4}$	0/4
131	*p*-Chlorophenol	$>10^{-5}$ $<10^{-4}$	0/3
140	Cotarnine hydrochloride	$>10^{-5}$ $<10^{-4}$	0/5
145	Thebaine hydrochloride (ethanolate)	$>10^{-5}$ $<10^{-4}$	0/4
192	Cinchonidine	$>10^{-5}$ $<10^{-4}$	0/3
11	*p*-Anisaldehyde thiosemicarbazone	$>10^{-6}$ $<10^{-5}$	0/3
35	α-Chloroacetanilide	$>10^{-6}$ $<10^{-5}$	0/4
98	4-Chlorochalcone	$>10^{-6}$ $<10^{-5}$	0/3
67	Di-*n*-octylamine	$>10^{-6}$ $<10^{-5}$	0/3
82	Narcotine	$>10^{-6}$ $<10^{-5}$	0/5
55	*n*-Lauryl thiocyanate	$>10^{-7}$ $<10^{-6}$	0/4
103	Pyronin B	$>10^{-7}$ $<10^{-6}$	0/6

* Concentration which causes 50 per cent inhibition of growth. When results with the two cell lines differed, that of the more sensitive cell was used.[4]

† Proportion of systems inhibited.

TABLE 1. Fourteen of these 20 compounds have been reported to exhibit definite antitumor activity against 2 or more experimental tumor systems. Four of these 14 compounds were cytotoxic against 1 cell line at 1.0×10^{-4} to 1.0×10^{-5} gm./ml., but were adjudged to be "culture-negative" (see above); 2 other compounds in this group could not be tested at concentrations of more than 1.0×10^{-5} gm./ml. because of insolubility.

Several compounds in this group (for example, urethan, formamide, and *N*-methylformamide) show some antitumor activity in man. The remaining tumor-active compounds not detected in tissue cell culture were effective only with a fraction of the experimental tumors tested. Short of study in man, there is no present means of determining whether it is the negative cell culture result or the occasional positive experimental tumor result that is valid in terms of activity in human tumors.

The reliability of the cell culture screen depends in large part upon the criterion used to assess "positivity." As illustrated in FIGURE 2, 78 per cent of all compounds cytotoxic at 1.0×10^{-4} gm./ml. were tumor active, 95 per cent of those cytotoxic at 1.0×10^{-6} gm./ml. were tumor active, and 100 per cent of those cytotoxic at 1.0×10^{-7} gm./ml. or less were tumor active. Of the latter group, 90 per cent were active in 2 or more experimental tumor systems.

Of the 91 compounds in the series that had no demonstrable effect on the experimental tumors tested, 19 (21 per cent) gave a false-positive result in tissue cell cultures, that is, they were cytotoxic by the arbitrary criteria used in these analyses. These 19 compounds are listed in TABLE 2. Again, there is no means of determining, short of study in man, which of these 2 results is valid in terms of the ultimate criterion, activity against human neoplasm.

Summary

One hundred and eighty compounds studied in at least three experimental tumor systems were assayed for cytotoxic activity in human cell cultures. There was no regular difference in the susceptibility of the cell lines derived from normal and malignant tissue to the various agents. The tumor-active compounds in the series were significantly more cytotoxic than the tumor-negative compounds. If 50 per cent or more inhibition of growth at 1.0×10^{-4} gm./ml. is used as the criterion of positivity, cell cultures detected 79 per cent of the 68 compounds in the series which have been reported to be active in two or more experimental tumor systems. Seventy-two per cent of these compounds were cytotoxic at 1.0×10^{-5} gm./ml., and 46 per cent were cytotoxic at 1.0×10^{-6} gm./ml. or less.

Of the 91 compounds in the series with no reported antitumor activity, 19 (21 per cent) were classified as positive, that is, were cytotoxic at 1.0×10^{-4} gm./ml. or less. Of these, 9 were cytotoxic at 1.0×10^{-5} gm./ml., 2 were cytotoxic at 1.0×10^{-6} gm./ml., and none were cytotoxic at less than 1.0×10^{-6} gm./ml.

All compounds cytotoxic at 1.0×10^{-7} gm./ml. or less were tumor active, and 90 per cent of these were active in 2 or more experimental tumor systems. Of the 37 compounds cytotoxic at 1.0×10^{-6} gm./ml. or less, 95 per cent were

tumor active, and 84 per cent of these were active in 2 or more experimental tumor systems.

These results justify a large-scale study of the potential usefulness of human cell cultures as a primary screen for the detection of antitumor agents. The observation that normal and tumor cells have hitherto responded similarly to a variety of antitumor agents in culture does not preclude the possibility that new agents eventually may be found that will be selectively active against tumor-derived cell cultures, and which, correspondingly, may be more effective therapeutic agents than the compounds now available.

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DISCUSSION OF PART II

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IRVING S. JOHNSON (*Biological Research Division, Eli Lilly and Company, Indianapolis, Ind.*): The papers presented in Part II of this monograph may be divided into two groups. Both Biesele and Eagle are concerned with the actual effects of drugs of potential usefulness in the treatment of cancer on cells in tissue culture. The differences between these papers are twofold. First, Eagle is working with established long-term cell strains and Biesele with short-term primary cultures that, in point of time, are closer to the *in vivo* disease in which we are interested. Second, Eagle obtains no selective effect with drugs upon the cell of malignant origin, while Biesele is able to achieve such an effect to a degree, but ascribes no significance to it. Whether this is an inherent difference between the types of culture systems or between the cells used remains open for discussion.

The first three papers may be grouped together as dealing with the nature of the cells that Biesele and Eagle are using in their tests. I should like to discuss two of these papers first. At present we are all intensely concerned with the importance of quotation marks. I refer to what amounts to their adverbial use to modify the adjectives "malignant" and "normal" as applied to cells in culture. We are all interested in learning whether these punctuation marks may be removed with any feeling of security. Moore has used successful transplantation, either homo- or hetero-, as a criterion for possible malignancy, followed by histological diagnosis from the *in situ* condition. While such diagnosis is of undeniable value, there remains the question whether all aspects of transplantation as a criterion for malignancy have been considered. I feel, as does Westwood,[1, 2] that "two cardinal criteria for malignancy are invasion and destruction of normal host tissue and production of metastases. The ability of cell lines to do this in animals whose defences have been destroyed by Xray or cortisone may no more demonstrate malignancy than the ability of normally commensal organisms to multiply in the tissue of an irradiated host demonstrates virulence." The chick embryo and the terminal cancer patient may fall into a similar category of hosts with little or lessened defensive mechanisms. Growth in these cases may serve only as biological profiles that may be used as a basis for further investigations. It is abundantly clear from the work of Toolan[3] that differentiated normal tissues also may grow in these conditioned animals. To the best of my knowledge, the fate of "normal" cells and tissues implanted in terminal cancer patients has not been investigated, but conceivably could be studied experimentally in animals. It seems to me that investigations of this type are fully as important as those carried out with malignant cells.

Leighton and Kalla have used an *in vitro* system that allows them to study one of the criteria for malignancy, that of invasion of normal host tissues. This is an admirable attempt to isolate a malignant reaction where it can be manipulated and studied in detail. One question in reference to this work occurs to me: in this system what would be the effect of any cell capable of rapid growth *in vitro*? While it is true that malignant cells probably multiply more rapidly than normal cells, certain normal cells, for example, such as those of bone marrow and regenerating liver, multiply very rapidly, indeed. In the case of long-term cell strains, we have selected for cells capable of rapid growth irrespective of "normalcy" or "malignancy." Many cell strains of normal origin multiply as rapidly *in vitro* as do strains of malignant origin, yet fail to cause tumors when inoculated into genetically compatible hosts. Moreover, these cells and cell strains of malignant origin that have either lost or declined in their tumor-producing properties when maintained under the same conditions as the normal cells have for the most part been ignored in this controversy. The behavior of these cell strains may well be of greater importance than the quotation marks that have been added to many normal cell strains.

Reserving comment on Foley's paper for the moment, let us consider again the effect of drugs in culture systems. The one discouraging result in Eagle's exhaustive drug survey has been the relative lack of any selective effect upon the cell of malignant origin. We have been testing drugs and antibiotic broths in a tissue culture system using long-term cell strains and, in our method at least, we see some selective action by known antitumor compounds.

One of my colleagues, Patrick Simpson, has been successful in adapting four human cell strains to the stirrer-type culture of Cherry and Hull,[4] with one important variation (FIGURE 1). These cultures are maintained in constant log phase of growth with no "gassing," and have an advantage over the conventional monolayer type in culture in the constancy of the physiological state of the cells at any given time. Two of the strains used are malignant in origin (HeLa and J-96) and two normal in origin (Earle's NCTC 2414 and LLC-H_1). Perhaps we naively tend to view the HeLa and NCTC 2414 as a normal-malignant epithelial system and the other two strains as a normal-malignant system of mesenchymal origin. Using the stirrer cultures as sources of seed, we have been inoculating duplicate flasks with replicate volumes of cells and measuring the original inoculum as well as the volume of growth during the 1-week test by the hematocrit method of Waymouth (FIGURE 2). This method embodies possible sources of error, but has the advantages of speed, ease, and reasonable reproducibility. The test medium consists of Eagle's basic HeLa medium, 10 per cent horse serum, and various concentrations of drugs or broths under study. The medium is changed midway in the test with the addition of fresh drugs or broths; a minimum of fivefold to sixfold multiplication in all untreated control cultures is considered as a prerequisite for a significant test. While the great majority of materials tested do not indicate any selective effect, certain drugs are capable of such action (TABLE 1). These examples of the type of effect obtainable in our system are reproducible in direction, although the magnitude may vary slightly.

While these results are not outstanding and their significance may be questioned by some, they do indicate that selective effects such as those seen by Biesele in short-term primary cultures also may be obtained with drugs against

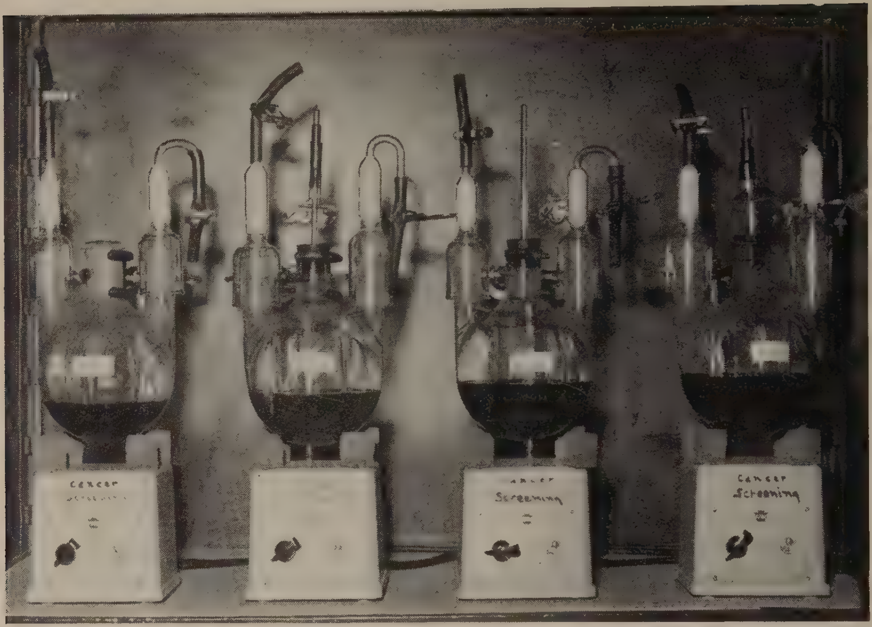

FIGURE 1. Four strains of human cells grown in stirrer culture with no gassing, used as a source of seed for routine testing of drugs and antibiotic broths for antitumor activity.

established long-term cell strains. Their magnitude is generally of the order of those obtained by the use of transplantable tumors.

This brings us to what I believe is the great significance of Foley's work with inoculation of cell strains into the cheek pouch of unconditioned hamsters, in which cell strains of normal origin fail to grow or regress and cannot be transplanted, and cells of malignant origin grow very well and are transplantable. Numerous investigators have cited the tendency toward polyploidy observable in long-term cell strains. Of course, this also occurs in malignant cells *in vivo* and is one of the causes of concern as to whether all such cell strains are malignant. Westwood studied 6 normal cell strains from rabbit, monkey, and man, 5 of which became transformed. All of these transformed strains, when injected into compatible hosts, conditioned or not, were unable to cause more than local tissue reactions, which regressed in from 8 to 10 days.

At first Westwood selected mutation (alteration of genotype by any one of a number of different mechanisms) as a more appealing explanation for these transformations than adaptation or the emergence from the primary cultures of an unrecognized but already present cell type. More recent studies have led him to postulate a genetic mechanism that involves a transfer in the control of basic metabolism from one gene locus to another without alteration of the genotype. There is ample precedence for this type of genetic mechanism in the control of the nature of ciliary antigens in *Paramecium aurelia* and the

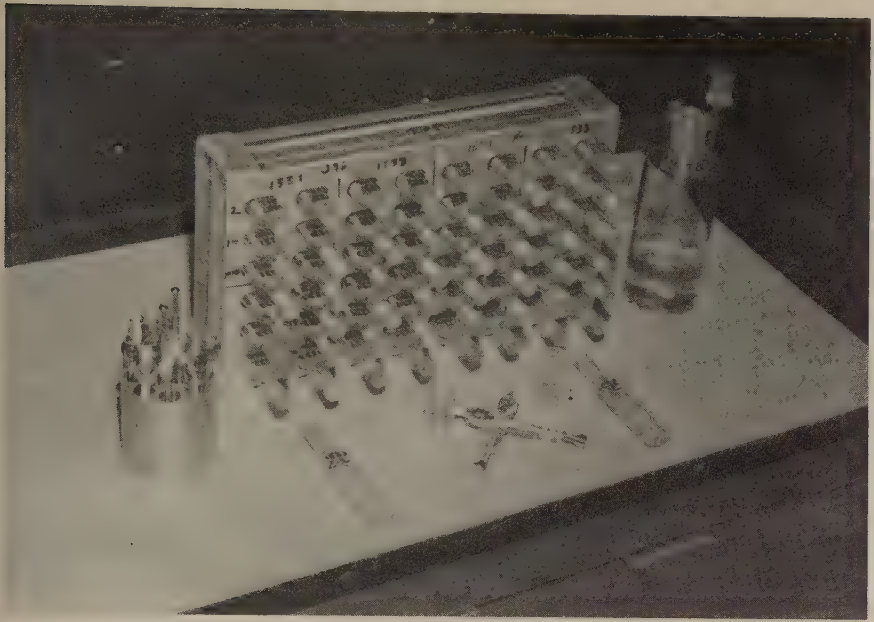

FIGURE 2. Flasks used for antitumor tests, hematocrit tubes, and a small stirrer culture maintained as an auxiliary for the larger cultures.

control of phase variation in *Salmonella*. Westwood has been able to demonstrate the extremely important phenomenon of reversion of the transformed state to the untransformed. The chromosome numbers of all cell lines examined by Westwood were found to lie in the hypotetraploid region regardless of whether the cells had undergone transformation. The acquisition of polyploidy followed as a secondary process to the adaptation of cells to the *in vitro* condition of growth.

Not being a geneticist and, indeed, at times slightly confused by genetic terminology, I feel that Westwood's work and that of Foley may illustrate in simple terms the following facts. When cells are put into culture there is a loss of functional stability. In general, cells *in vitro* no longer are capable of performing some of the functions they did perform *in vivo*. In this artificial condition these cells have been removed from the control of any systemic normal regulatory mechanism for cell division. By the nature of tissue culture techniques, selection is made for the cell capable of rapid growth, which in some way is represented by the polyploid cell. In the case of the normal cell, however, sensitivity to the normal regulatory mechanism may still be retained. When Foley inoculates these normal cells into the unconditioned hamster cheek pouch he may be returning them to the authority of the normal regulatory mechanism. The malignant cell is no longer sensitive to this mechanism, which is only a restatement of a generally recognized phenomenon of malignancy, namely that of autonomy.

TABLE 1

EXAMPLES OF SELECTIVE INHIBITION OF THE GROWTH RATE OF A CELL STRAIN OF MALIGNANT ORIGIN AS MEASURED BY THE HEMATOCRIT METHOD BY FIVE KNOWN ANTITUMOR COMPOUNDS

Drug	Conc. in γ/ml.	Percentage of inhibition			
		HeLa	NCTC 2414	J-96	LLC-H$_1$
Methotrexate	2.5	No selective effect		86	83
	0.025			88	30
	0.0025			21	0
6-MP	3.0	100	100	100	78
	0.03	68	31	56	24
	0.003	50	0	21	28
Azaserine	1.0	100	92	No selective effect	
	0.01	36	0		
	0.001	26	6		
5-Fluorouracil	6.0	96	81	No selective effect	
	0.06	62	46		
	0.006	42	4		
TEM	1.0	No selective effect		100	100
	0.01			37	0
	0.001			6	0

I think it is only natural to expect that much of the future work in cancer research will be conducted with established cell strains produced in large quantities by one or more of the technically more efficient systems of cell production described in the past few years.[4-8] Work with the systems available to us must be done cautiously and with constant reflection and consideration. We should not let ourselves be stampeded by punctuation marks. While pursuing these newer lines of endeavor in tissue culture, we should not ignore broadened explorations along more conventional lines, particularly in the functional stabilization of cells in culture in terms of the *in vivo* state.

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EAGLE: Although, in our last study, Foley worked with one cell line and I with another, independently, he in the normal and I in the malignant cell line, there were as many as threefold to tenfold differences in the susceptibility of compounds of these cell lines to the agents we studied. I regret to say, however, that in more than 97 per cent of the cases it was the cell deriving from normal tissue that was the more susceptible.

GEORGE O. GEY (*Finney-Howell Cancer Research Laboratory, The Johns Hopkins Hospital, Baltimore, Md.*): I agree with Johnson, with Biesele, and with others who contend that we should stay as near to the patient's problem and to the patient's tissue situation as possible. I believe this is indispensable. Perhaps we should always consider harvesting the patient's specific normal tissue and the derived tumor cells simultaneously and try to put them into an environment where they can be observed during the period when these various wonderful compounds may be given an opportunity to act differentially upon them.

The heterologous transplantation systems featured in the excellent work of David Karnofsky and of Helene Wallace Toolan have given us valuable information; they remind me of earlier experiences of thirty-odd years ago when I learned that some human normal and malignant tissues (especially placenta) live for several weeks in the peritoneal cavity of the horse. Shirai's technique for brain implantation, as revealed in the excellent work of Greene, has provided a good model for the study of normal and malignant tissue of isologous origin, permitting conjoined activity for weeks, during which a suitable challenge can be made with any available compound. This can be done in the mouse.

Our experiences with continuous tissue cultures of more-or-less stabilized strains have taught us that we can have isologous normal strains differing from their tumorous tissue culture derivatives. However, when the tissues or the dispersed cells are grown in strange media at very rapid rates, many investigators have found new properties in these derived cells (which are now so altered that they differ considerably from their original normal state). Such experiences emphasize the need for more work that might contribute to a better understanding of growth regulation and the maintenance of the homeostatic mechanisms so necessary in the preservation of our somewhat elastic normal physiological state. We have yet to learn how to maintain many of our cells in culture as stable normal strains. Sometimes after we have established a fairly stable normal strain, let us say for 16 years, we farm it out to another experienced investigator. This investigator grows it in a new concoction for an extended period, during which it undergoes profound alteration, and then sends it back to us in unrecognizable cell form. Frequently we have received the impression that the cell shipped back to us has come from some other host. Now that Parker *et al.*[1] have described what happens, we shall attempt to prove our point, for we have here either a new dramatic transformation or an accidentally acquired contaminant substituting for our strain 14p. In our hands, with but one exception,[2] the 14p strain has remained quite stable for almost 20 years.

On the basis of these findings, we may say that many of the cell strains maintained today in a variety of media undoubtedly represent somewhat diverse cell types. The exact condition of what is being maintained as the HeLa strain might prove interesting. We already know of the existence of specially selected HeLa strains[3-5] with both narrow and broad variations in chromosome number. These and other strains show considerable differences in nutritional properties and viral susceptibility. Our parent strains of HeLa do not resemble some of the stock strains I have seen. These morphologic comparisons, however, may have been much too superficial. We are trying to maintain some parent strains in strictly homologous media. Here they show great changes—morphologic and chromosome aberrations familiar to all investigators.

Differences between stable normal strains such as our rat strain 14p and its tissue culture derivative, the tumorous cells T-333, have considerable meaning,[2] although other investigators may not stress the differences known to exist. In some instances we have had experiences similar to those of our good friend Federoff, at Saskatoon, Saskatchewan, Canada (Federoff and Hoffer[6]). Federoff has been exposing altered tumorous cells such as Earle's L strain to certain schizophrenic sera. In this case the poor defenseless L strain dies quickly. Certain apparently normal adult sera also kill the L strain—an experience that we and many have had in work with adult sera and various other cell lines.

Other comparisons between normal and derived altered normal cells and tumorous cells show interesting results. When we try to grow our rat tumorous strain T-333 in human adult sera at a 50 per cent dilution with BSS and without the support of a synthetic medium, it is soon killed, whereas the specific normal prototype thrives. We have observed these results sufficiently often to convince us that a great deal can be learned by examining these sera for inhibiting polysaccharides, as Federoff has begun to do.[7] We may also learn a great deal from the host, should the contributing factors, in the form of amino acid deficiencies or other factors, originate there.

Undoubtedly more effort is being, and should be, placed on attempts to establish isologous normal and malignant cell strains. We have observed a dozen differences in the 14p and T-333 strains, including differences in cellular constitution, nutritional requirements, metabolic patterns, and viral susceptibility. Some of the reports included here on tumor cell lines and other rapidly growing, possibly altered, strains deriving from normal tissues provide important information on the similarities and differences in nutritional requirements and responses to drugs. As Eagle and others have pointed out, it is of utmost importance to know if this is the situation in a normal host. Evidence points significantly to the probability that we shall encounter altered cells soon after the primary cultivation, especially when such cells are grown in certain media.[1, 8-10]

Kent and I[11] have tried to learn something of the responses of some of our cell strains to various protein moieties, and we hope to ascertain differences between normal and malignant cells. These studies of protein depletion are enabling us to understand more clearly the process of release or utilization of the amino acids from these proteins during growth. We recognize that this differs widely from the host situation, where many of our normal cells are firmly bound up in an intercellular matrix from which they do not easily migrate, especially in some adults.

I am delighted with the progress of such investigators as Leighton[8] and Wolf and Schneider,[12] who have been observing the interactions in culture between various normal and malignant cells. These easily manipulated systems already show promise by reviving much of the interest formerly shown in cellular interactions.

Regarding our tissue culture responses in general, especially as they relate to isolations from human sources, a large proportion of human tumors cannot be isolated as stable strains of specific histogenic origin, whether we use the hamster cheek-pouch technique, the irradiated host (Toolan), or direct tissue culture isolation. We must learn much more about the nutritional and hormonal requirements. Results have begun to appear in this area at the cellular level.[13] Such information can be applied to our chemotherapy researches.

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Lawrence Berman* (*Department of Pathology, Wayne State University College of Medicine, Detroit, Mich.*): Although this monograph is devoted to screening procedures for experimental cancer therapy, the topic of this part implies that there are unsolved problems concerning the normal or malignant nature of the cells we should like to use in our experiments. Much of the recent work with cultured cells is based on techniques lately developed by virologists.

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These have been designed to promote maximum proliferation of cells. The methods are quite different from the classic plasma-clot techniques of tissue culture. It is doubtful whether we can apply all of our past experience with tissue culture to the present type of cell culture, which has the practical aim of obtaining large quantities of living cells, rather than the study of the cells themselves. Even those who have no close alliance with the business of screening drugs find it easy to become preoccupied with the activities of the stable cell types, which seem to have become tools in the hands of virologists and pharmacologists.

There now appears to exist a body of evidence indicating that cells of normal mammalian tissues, cultivated by the present widely used microbiological methods, undergo changes of a characteristic pattern.[1-4, 8, 11] The original cells of the explants are succeeded by one or more phases in which other types of cells predominate; these are usually different from any of those present in the original cell population. There may be a fibroblastlike (FbL) cell phase of temporary duration, which is then replaced by the sudden appearance of an epithelial-like (EpL) cell phase. The change in the FbL cell layers occurs focally.[4] The foci of polygonal cells enlarge and, on repeated subculturing, the EpL cells persist as the only remaining elements.

Concomitant changes in morphology, transplantability, and chromosomal constitution have led to a widely held notion that the cells may have become malignant *in vitro*.[4, 5, 7, 9] This is a problem of obvious interest to the cancerologist desiring to use such cells for screening experiments and to the virologist engaged in the preparation of vaccines for human use. Nevertheless, to one curious about the cells rather than their application to specific uses, transformation from the normal to the malignant state appears less spectacular than the transformation of the original cells of the explants to the EpL cells of the stable strains. To an outsider, the use of such cells as prototypes or experimental models seems to require a great deal of intelligent intuition.

In the laboratories of the modern physicist, new elements that do not exist in nature are produced experimentally. We might wonder whether we are doing something analogous, in the manner of biological alchemy. It would be very helpful to know whether the cells of stable strains are more simplified, or more complicated, or unchanged except for superficial appearances. If they are simpler, we may have at hand the means for studying cell functions that are otherwise obscured by complicating and perhaps conflicting activities. If they are more complicated, we have at hand examples wherein latent potentialities of cells, not expressed *in vivo*, become exposed *in vitro*, and part of our attention might be directed to the mechanisms that regulate or inhibit the manifestation of potentialities not easily observed *in vivo*. If they are unchanged except in morphology, we may be able to decide whether or not morphologic study of fundamental problems at the cellular level is no more than a waste of time.

Investigations carried out by Cyril Stulberg, Frank Ruddle, and myself illustrate all these paradoxical possibilities. For example, when the EpL cells of the Detroit strains of human origin are made to proliferate on glass or in sponge-matrix cultures they form sheets or masses of completely undifferentiated character. However, when cells such as EpL cells from normal human bone marrow are grown in the chorioallantoic membranes of chick embryos, they form cords or tubular structures composed of cuboidal cells laid on distinct basement membranes.[4] The experiment exposes morphogenic potentialities that do not occur among cells of intact human bone marrow.

On the other hand, EpL cells derived from human sources as widely divergent as normal peripheral blood and carcinomatous ascitic fluid have similar appearances and growth behaviors in established cultures. Even the degree and nature of chromosomal changes are similar among the various EpL strains, whether derived from normal or from malignant sources. The chromosome numbers of strains from normal human blood, as is also the case in strains from malignant tumors, usually fall within the range of 65 to 80, with occasional normal blood cell lines having modal values about double this number.*

Nevertheless, Foley and Handler[6] have demonstrated that, despite morphologic similarities and, as far as the Detroit strains are concerned (personal communication), chromosomal similarities, the transplantabilities of different EpL cell lines into unconditioned hamsters may differ. There is also some correlation between transplantability and the source of the cells, that is, whether the cells emerged from normal or malignant sources. This is at least suggestive of the idea that the condition of heteroploidy per se is not the factor that makes the cells transplantable. The work suggests that it may be necessary to explain the observed differences in transplantability on the basis of attributes other than the genetic or chromosomal constitution of the cells. Recently there has been a great increase of interest in the chromosomal constitution of mammalian cells in cultures; many investigators are attracted by the concept that normal tissues become malignant when they change their chromosomal constitution. Hsu[7] cautiously warns against early acceptance of this oversimplified conclusion. His studies, as well as our own, lead to one safe conclusion: that all "normal" cell strains studied are abnormal with respect to their chromosomal constitution.

Finally, some of our recent studies of viral susceptibilities seem to indicate that there are important relationships between form and function of cells in culture. In a series of publications we have already indicated that there are some definite morphologic phases in the development of EpL cell strains. In phase III, which is temporary, the cells have a fibroblastlike form. In some cultures this FbL phase is followed, after variable periods of proliferation and usually after a temporary period of slow growth, by a fourth phase, the typical EpL phase of most stable human cell strains. In one of our cultures from a lesion of urticaria pigmentosa we obtained an FbL strain that gave rise to polygonal cells in several bottles of the sixth serial subculture. These outgrew the FbL cells; thus, a substrain of EpL cells that was maintained by serial subculture emerged. Meanwhile, in parallel cultures, EpL cells did not appear, and cells serially subcultured from these have maintained their FbL morphology for more than a year. This gave us the opportunity to study the viral susceptibilities of an FbL strain and an EpL derivative of the same culture. The FbL strain supports the growth of certain ECHO viruses that do not proliferate in the EpL variant.[10]

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* F. H. Ruddle, L. Berman and C. S. Stulberg, unpublished observations.

There are a number of additional and obvious differences among cells of the FbL phase and cells that may emerge from this phase to become EpL. Although we have been able to preserve and recover all of our cell strains, both FbL and EpL, by a method of slow freezing at controlled rates, there was a considerable difference between the two cell forms when rapid methods of freezing were applied. The EpL cells could be recovered successfully, but the FbL cells were quickly destroyed.*

An important by-product of our present preoccupation with cells for testing chemotherapeutic agents is of a dual nature: (1) it opens a way for the investigation of interrelationships between morphology, transplantability, chromosomal constitution, antigenic specificity, and viral susceptibilities; and (2) a matter of even more fundamental importance, it shows the need for investigation of the mechanisms of cell transformations of any kind occurring *in vitro* With the second of these general approaches in mind, we have begun to culture normal human peripheral blood because it contains only two cell types capable of mitosis or further differentiation, lymphocytes and monocytes. Phase I of the cultures is characterized by the presence of identifiable leukocytes. Later the cultures are composed of large round cells (polyblasts, mononuclear cells, histiocytes, epithelioid cells of the older tissue culture literature, and phase-II cells in our terminology). Finally, we end up with sheets of polygonal EpL cells that can be maintained indefinitely thereafter. They cannot be distinguished morphologically from cells derived from nonhematopoietic tissues. They no longer have the appearance of cells of blood or hematopoietic tissues. They no longer have the chromosomal content of normal human cells. They are heteroploid and possibly malignant.[1] Excluding the question of malignant transformation, these events, to say the least, are astonishing. If we consider cell size alone, we have a formidable bit of explaining to do. The EpL cells have nuclei that are sometimes ten times larger than the nuclei of large lymphocytes, when both are spread on glass and firmly attached. In some EpL cells the nucleoli alone may be larger than the entire nuclei of the largest lymphocytes or monocytes of the original blood cell suspensions.

Of course there are many very important uses to be made of these interesting monsters developed in the laboratory, as is so well illustrated in this monograph. The study of the transformation from the original state to the stable EpL state is a subject some of us will not be able to pass over without being profoundly stimulated—a matter of importance to those who are interested in the cells rather than the uses to which they may be put. This, however, may be a subject worthy of another monograph.

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Eagle: If taken literally, some of the discussion would lead one to believe that the study of cells in culture is to be undertaken in place of the study of the behavior of cells in tissues *in vivo* or instead of the study of the interaction between cells. Insofar as I am aware, this has never been intimated even by those interested in cells. These approaches are not usually exclusive; usually they are supplementary.

Has Leighton had an opportunity to explore in his *in vitro* system the relative invasiveness of clones of, let us say, the HeLa cell, which differ remarkably from some of the others with respect to their ability to migrate on the surface of glass? Some of these cultures form very compact and others very diffuse clones in which the cells have or have not an extraordinary capacity to move on the surface of glass. I wonder if Leighton has had an opportunity to compare these different types of clones.

Joseph Leighton (*University of Pittsburgh School of Medicine, Pittsburgh, Pa.*): We worked very little with cloning techniques, and what we have done with them has been done primarily with D-189. In this cell line we find the same variations that you described for HeLa, that is, some very tight groups of cells and others that spread out greatly. We shall be comparing these two kinds of patterns in interaction systems later on.

Question: When you showed the normal requirements of amino acids for epithelial cells versus malignant epithelial cells, did you include any amino acids that are used over the fibroblasts?

Eagle: In the case of the fibroblast, we have examined the Earl's 929 L cell (if that can be called a fibroblast), the S-180 culture, tumor cultivated by Foley, and a human fibroblast, the MAF line isolated by Gray at Microbiological Associates, Inc., Bethesda, Md. All three of these cells had the same amino acid requirements as all of the others in the epithelial type cell study. As far as the utilization is concerned, with the exception of only glutamine, none of the essential amino acids is metabolized to an important degree. They are all used primarily for the protein synthesis, with very little indication of degradative metabolism.

Leighton: Has Foley yet had an opportunity to take some of the normal cells that fail to grow as tumors in a cheek pouch and supplement them with any sort of embryonal stroma in this site? In other words, has he been able to repeat the kind of thing Green has done for the guinea pig eye? Do you then think that some of these cells might produce significant nodules under these circumstances?

G. E. Foley (*Children's Cancer Research Foundation, Inc., Boston, Mass.*): We have not yet done the experiment.

Comment: The word "chromosome" reminds me of a more recent experiment with the *in vitro* tissues that undergo heterotransformation or chromosome alteration. This particular process also occurs in one of the *in vivo* cultures. What we see as characteristic for *in vitro* is not necessarily peculiar to *in vitro* environment, but also can occur in the well-established *in vivo* lines of the tumor. The mechanisms and the indications are quite comparable, once we consider that both are proliferating tissues in cultured media, synthetic or partially synthetic.

Comment: Are the people in Washington, D. C., or any other investigators, planning to repeat something done a few years ago, namely to determine which method would be best for concentrating the substances of antineoplastic or neoplastic activity on the basis of another biological activity? This would now require the use of literally thousands of substances and double blank tests with many different preliminary screenings. I think your studies point to one method of testing activities at very low concentration. I am not sure about the numerical data. For instance, if we isolate only the ones that are active at up to 10^{-6} gm., how many would there be among the group of 200 and how many of this group would be antineoplastic? I think we should not look too hard to find the method that would isolate from a group of thousands of substances, all antineoplastic. It would be much better to find a method that would isolate 10 substances, half of which would be antineoplastic, if we included 50 or 60 per cent of others.

Eagle: Your point is well taken. In screening we cannot afford to worry about the compounds we have missed. We must keep in mind the compounds we have detected and the possible significance of their positive result. I think the Cancer Chemotherapy National Service Center does contemplate a large-scale study in which some thousands (rather than hundreds) of compounds and antibiotic filtrates will be tested in parallel by the conventional animal tumor screens, by the microbiological screen discussed in Part I, and by the cytotoxicity screening in cell cultures discussed here in Part II. All of these compounds will be submitted under code number.

Question: I should like to ask Berman by what criteria these fantastically normal cells, each deriving from cultures of normal nodules, are epithelial type cells. Why are they not used with the fibroblast cells or the genital cells, or something like that?

Berman: With regard to the first question, I want to point out that the terminology in present-day cell culture is something that grew out of a more or less missuse of terms by many of us who have appropriated old words for new meanings. Epithelial, in this sense, is merely a device to save time and descriptive efforts. It means cells that grow more or less like pavement epithelium, usually polygonal cells closely juxtaposed to each other. It has no significance with respect to the question of whether they are truly the kind of cells that would form epithelial structures *in vivo*; and it does not mean that they come necessarily from epithelium *in vivo*. Similarly, "fibroblastlike" is a term that applies to the appearance of the cells. It does not mean that we have evidence of proof that the fibroblastlike cell is, in fact, the same kind of cell that forms, for example, collagen in the body.

QUESTION: We have here overwhelming evidence for change in the cultures currently available. I wonder if anyone would take the opposite side, someone who has found some stability in the material with which he has been working.

EAGLE: Biochemically, in terms of all the criteria examined to date, these cells are stable in spite of their obvious changes. Only some of them are detailed here. These cells have changed in many other aspects but, with respect to their nutritional requirements and what they use in this connection or how they use their nutrilites. They are not only remarkably uniform, but also remarkably stable.

Part III. Ascites Tumors

ASSAY OF MITOTIC POISONS ON THE HYPERDIPLOID EHRLICH ASCITES CARCINOMA

By Hans Lettré

Institute for Cancer Research, University of Heidelberg, Heidelberg, Germany

In 1932, Loewenthal and Jahn[1] reported on the transformation of the solid Ehrlich mouse carcinoma into an ascites form. In 1939 I started investigations on the action of chemical compounds on normal and malignant cells grown *in vitro.* In connection with these experiments, I considered making use of a form of tumor growth in the animal that would allow a comparison with the conditions of the tissue culture. Loewenthal and Jahn,[1] as well as Collier and Jahn,[2] had claimed that the ascites form of the Ehrlich carcinoma was chemoresistant. Our experiments demonstrated, however, that this ascites tumor is an extremely sensitive indicator for the action of a suitable chemical factor.[3] We began transferring this line of the Ehrlich ascites carcinoma in 1940. Because of postwar technical difficulties, the line died out in my laboratory in the cold winter of 1946–1947, although it was renewed from samples of the same line given previously to other laboratories.

In 1952, Bayreuther[4] showed that this tumor line is hyperdiploid. In this respect, our tumor line differs from another ascites form of the Ehrlich carcinoma developed by Klein *et al.*,[5] which is tetraploid.[6, 7] Our reports refer only to the hyperdiploid tumor and to sublines developed from it.

The problem was to find conditions that would permit use of this tumor in a quantitative test method. The characteristic feature is the development of a large amount of ascites after intraperitoneal injection of the tumor cells. The total weight of the inoculated mouse is increased by the growing tumor and the production of ascites. In FIGURE 1, two typical weight curves of such inoculated mice are shown.[3] Several days after injection the curve increases, reaches a maximum value, and decreases until the animal dies. The maximum value may not always be observed, as the animals may die during the phase of rapid growth of the tumor. In some cases the animal's weight may decrease for a short period immediately following injection. Alternatively, the increase of weight may be as much as 20 gm. or more.

The weight curve is the result of at least three factors: (1) the weight increase of the growing tumor cells, which are located mostly in the ascites, but also partly invade the organism; (2) the weight of the ascites; (3) the change of weight of the host animal itself. The increase of weight by the tumor is relatively small, of the order of 1.5 to 3 gm., averaging approximately 15 per cent of the daily weight increase (10 gm. of tumor ascites gives an average of 1.5 gm. of wet residue after centrifugation). The quantity of tumor cells inside the organism can be ignored during the period of development of the ascites tumor. The change of weight of the mouse itself is not decisive. Reweighing the mice after their death and after removal of the ascites has revealed de-

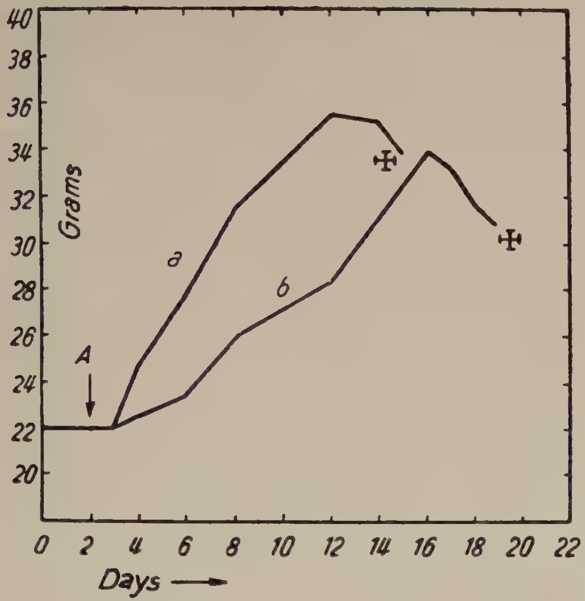

FIGURE 1. Weight curves of mice inoculated I.P. with 0.2 cc. tumor ascites at *A*.

creases of only 2 to 3 gm. If we examine the body weight at different times before death we find increase as well as decrease of the body weight, resulting from nutrition or other conditions. Actually, the decisive factor in the weight curve is the weight of the ascites, although the increase of the ascites is proportional to the multiplication of the tumor cells and is therefore the enlarging projection of the small increase of the weight of the tumor cells themselves.

We have counted the numbers of cells in the ascites at different times after inoculation.[8] During the phase of rapid development of the tumor the number of cells as well as the amount of ascites increases. Before reaching the maximum, however, the ascites formation prevails over the increase of cell number. TABLE 1 shows the daily weight increase of 200 mice inoculated with 0.2 cc. tumor ascites.[3] The daily weight increase is the quotient of maximum minus starting weight through the days from inoculation to maximum time. The result is a typical distribution curve with a prevalence of values from 0.4 to 1.2 gm./day.

The life expectancy of the mice after inoculation is 12 to 16 days. TABLE 2 shows the survival times of 200 mice inoculated with 0.2 cc. tumor ascites. From these values we can calculate the life expectancy of 50 per cent of the mice as 14.2 days (values from experiments of the year 1940[3]).

TABLE 3 shows a summary of the values observed with animals of the control groups of experiments we made in the years 1940, 1941, and 1949 to 1957. For each year we have calculated the survival time of 50 per cent of the animals (S_{50}). Generally, the yearly results average 14 days, taken from the values of 3207 animals. The greatest deviations occurred in the years 1951

TABLE 1

DAILY WEIGHT INCREASE OF 200 MICE INOCULATED I.P. WITH 0.2 CC. TUMOR ASCITES EACH (1940)

Weight increase (gm.)	Number of animals	Weight increase (gm.)	Number of animals
0.0–0.3	None	1.2–1.4	19
0.3–0.4	12	1.4–1.6	6
0.4–0.6	31	1.6–1.8	3
0.6–0.8	47	1.8–2.0	1
0.8–1.0	44	2.0–2.2	1
1.0–1.2	36	2.2–2.4	None
Total.	. . . .	. . . .	200

TABLE 2

SURVIVAL TIME IN DAYS OF 200 MICE AFTER I.P. INOCULATION OF 0.2 CC. TUMOR ASCITES EACH (1940)

Days	Number of animals	Days	Number of animals
1	none	13	28
2	none	14	33
3	none	15	37
4	none	16	27
5	none	17	15
6	2	18	8
7	3	19	8
8	3	20	5
9	1	21	3
10	4	22	3
11	6	23	1
12	13	24	none
Total.	. . . .	. . . .	200

TABLE 3

HYPERDIPLOID EHRLICH ASCITES CARCINOMA

Year	Number of mice	S_{50}* (days)	Year	Number of mice	S_{50}* (days)
1940	200	14.2	1953	464	14.2
1941	102	13.7	1954	542	14.6
1949	250	11.8	1955	295	13.7
1950	277	14.2	1956	339	13.5
1951	217	16.3	1957	89	13.3
1952	432	15.0			
				3207	14.0

* S_{50} = period in days during which 50 per cent of the animals died; dating from the years 1940, 1941, and 1949 to 1957.

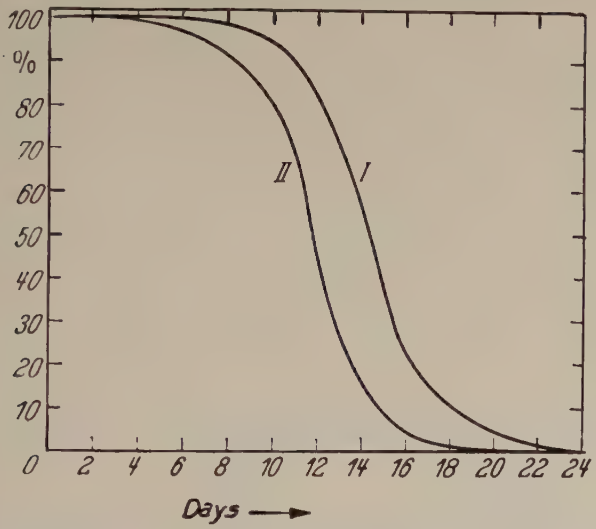

FIGURE 2. Life expectancy of mice each inoculated I.P. with 0.2 cc. tumor ascites. Curve I was derived from 200 mice (1940); curve II, from 250 mice (1949).

($S_{50} = 16.3$) and 1949 ($S_{50} = 11.8$). FIGURE 2 shows the results of the years 1940 and 1949 in curves.

Which factors influence the type of the weight curve? In 1940, in order to examine the influence of nutrition, I performed an experiment in restriction of diet.[3] The results are shown in FIGURE 3. Curve I represents the weight of mice with normal diet after the inoculation of tumor cells; curve II the weight of animals without tumor on normal diet. Curve IV represents normal mice starving for several days and, at B, feeding with bread for one day. There is a decrease of weight and then, after refeeding with normal diet, a return to normal weight. In curve III, representing the same procedures as in IV, using animals with tumor, we see the same type on "changing weight curve." After the refeeding on normal diet, however, the animals not only regain weight, but also the ascites tumors develop, which leads to the death of the animals, with some delay in comparison with the control group (curve I). While this is a drastic experiment, smaller variations in the diet also might influence the character of the weight curve. In 1949 it was again possible to give our animals milk in addition to their regular diet of bread, cereals, and water. This caused an increase in the quantity of ascites.[9]

The Ehrlich ascites tumor is nearly 100 per cent effective in producing takes by injecting 0.2 cc. tumor ascites; since the takes are not dependent on the strain of mice, stock mice can be used for all experiments. The type of weight curve is different with different strains of mice, however.[3] Injection of tumor ascites from one mouse to mice of different strains (db mice, mice of a black strain, and white stock mice) produced the different weight curves shown in FIGURE 4. Obviously, for experimental work (for instance, screening proce-

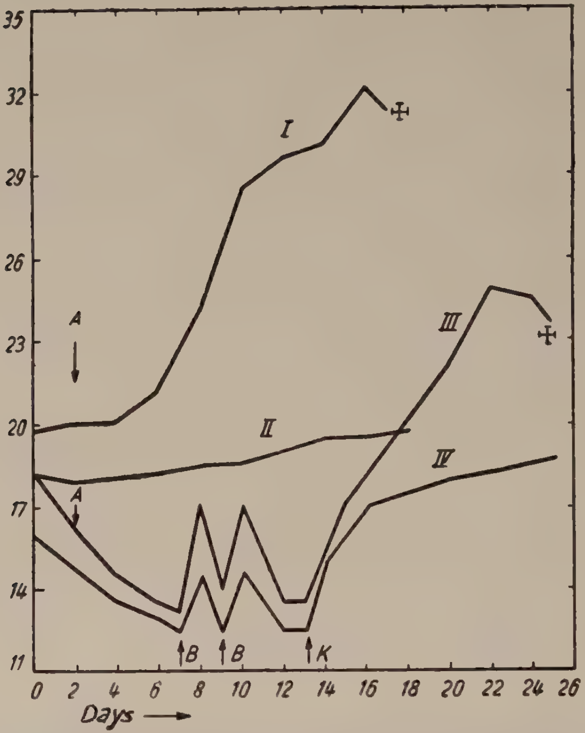

FIGURE 3. Effect of diet on weight curve. Curves I and II represent mice receiving normal diet (mice of curve I inoculated with 0.2 cc. tumor ascites at *A*); curves III and IV, mice receiving greatly reduced diet (mice of curve III inoculated with 0.2 cc. tumor ascites at *A*). In III and IV, mice received one day's feeding of bread at *B*, and normal diet at *K*.

dures), animals of the same type must be taken and maintained under the same conditions. Naturally the speed with which the tumor grows is also a function of the number of inoculated cells.

FIGURE 5 shows weight curves of mouse groups inoculated with 64 million (I), 16 million (II), and 4 million (III) tumor cells.[3] The diminution of numbers of transplanted cells results in an elongation of the latent period until commencement of weight increase and an increase of survival time. The simplest assumption about the type of the multiplication of tumor cells is that the increase of cell number is proportional to the number of tumor cells present. This leads to the equation

$$Z_t = Z_0 \cdot 2^{t/t_M}$$

Here, Z_0 is the number of cells at time 0, that is, the number of inoculated cells; Z_t, the cell number at the time t in days; and t_M, the doubling time of the number of tumor cells.

From the values of the experiment shown in FIGURE 5, we can calculate values of 2 to 2.5 days for t_M. Another calculation based on the quantity of

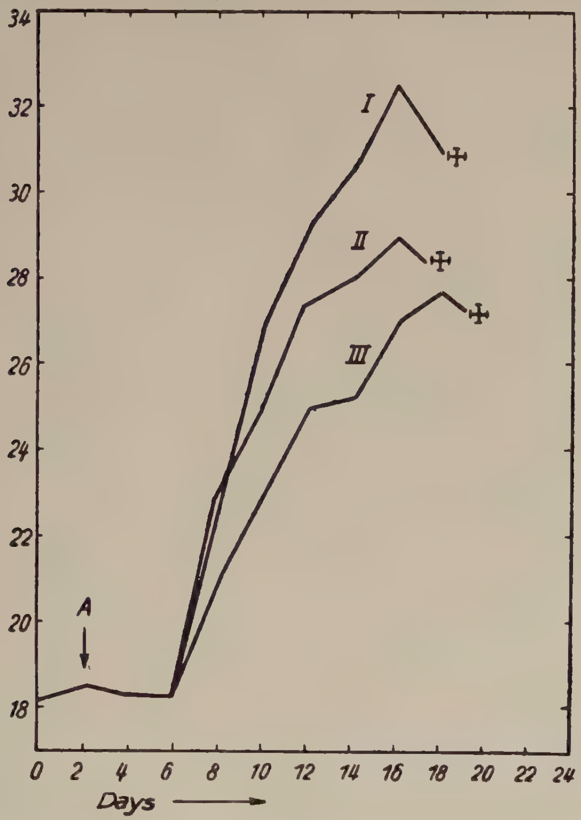

FIGURE 4. Weight of mice inoculated I.P. with 0.2 cc. tumor ascites. Curve I represents dilute brown strain; curve II, black strain; curve III, white stock mice.

ascites leads to similar values.[10] This doubling time of 2 to 2.5 days will be used to calculate the time required by a single tumor cell to multiply into an observable ascites tumor, that is, about 64 million cells. This would require approximately 26 times t_M (2^{26} = about 64 million), which would be 52 to 65 days. Only after this time can an animal be said to represent a negative value.

These results gave the basis for the development of the following testing method.[3] The mice used are of the same origin, with a practically constant weight of 20 gm. or more (mice of a lower weight are not desirable because the increase by ascites production is greater with animals of greater weight). These mice are kept in groups of five each. One group receives tumor only, a second group receives, in addition to the same quantity of the same tumor cells, the compound to be tested. Although the individual weight curves of the mice used might differ, the average of a group of five mice has been found to be comparable with that of another group of five mice. This method, determined by experience and not by biometric calculation, proved to be correct in about five thousand experiments.

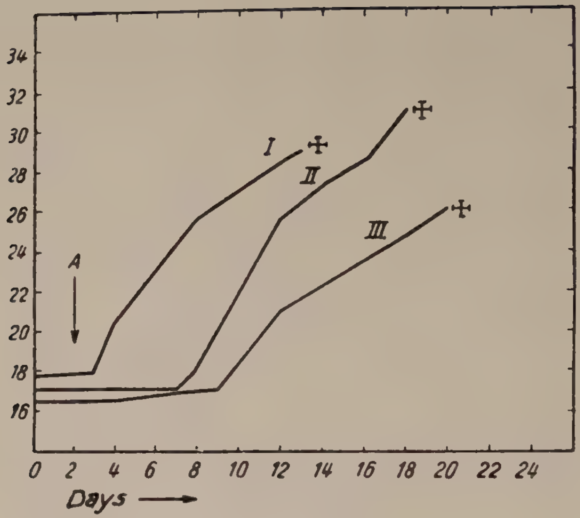

FIGURE 5. Weight curves of mice inoculated I.P. at *A* with: curve I, 64 million tumor cells; curve II, 16 million tumor cells; and curve III, 4 million tumor cells.

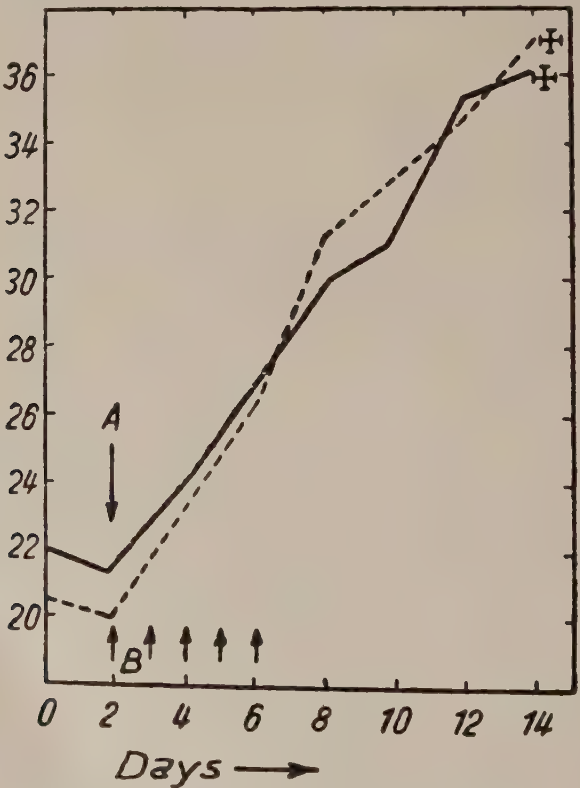

FIGURE 6. Comparison of weight curves with mice inoculated with an inactive solution. Dotted line = control, mice inoculated I.P. at *A* with 0.2 cc. tumor ascites; continuous line = mice inoculated I.P. at *A* with 0.2 cc. tumor ascites and, at *B*, with injection of 0.5 cc. physiological saline solution.

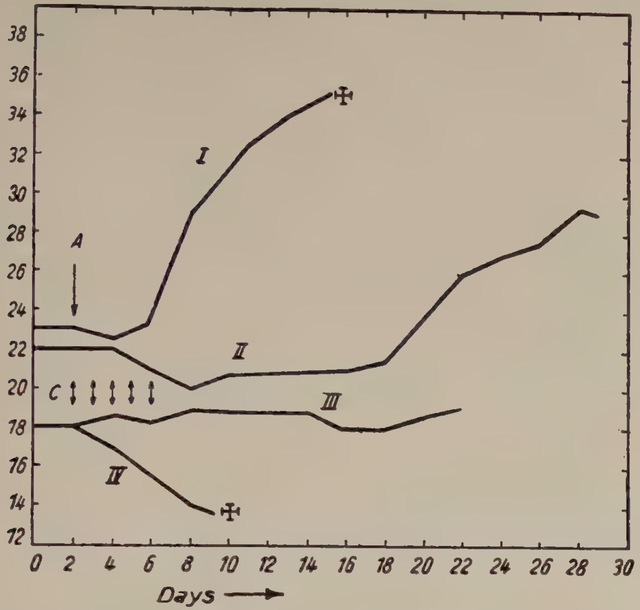

FIGURE 7. Weight curves comparing effective compounds. Curve I, I.P. inoculation of 0.2 cc. tumor ascites at *A*; curve II, I.P. inoculation of 0.2 cc. tumor ascites at *A* and, starting at *C*, of 10 μg. colchicine daily for a period of 5 days; curve III, only 10 μg. colchicine as in curve II; and curve IV, 20 μg. colchicine daily for a period of 5 days.

FIGURE 6 shows an example of the use of a solution assumed to be inactive, such as physiological saline,[3] demonstrating the equality of the type of the weight curve of both groups and also of the survival time. In the case of an effective compound, however, we can observe the difference in the weight curves of both groups, as shown in FIGURE 7 for colchicine.[3] Curve II demonstrates the striking difference as compared with curve I of the control group. The compound is administered on five successive days, starting with the day of inoculation. Because of the pressure of the taut peritoneum, an intraperitoneal injection into an already developed ascites tumor cannot be performed without loss of ascites. This treatment is usually given only in cases where a compound is ineffective at the sixth day. Where the compound is effective, the injections can be continued, but for the sake of comparability we stop these also on the fifth day.

The difference of the weight curves gives the first hint as to the effect of a compound. It must be remembered, however, that this difference is not due to the toxic effect of the compound. For this reason, a third group of mice receives the compound alone, without inoculation of tumor ascites. For example, in FIGURE 7 the daily injection of 10 μg. of colchicine does not influence the weight of such a group of mice (curve III), but an amount of 20 μg. causes the animals to lose weight and die (curve IV).

Observation of the weight curves during an experiment in the first eight days reveals the possibility of interpreting as negative the cases where there is

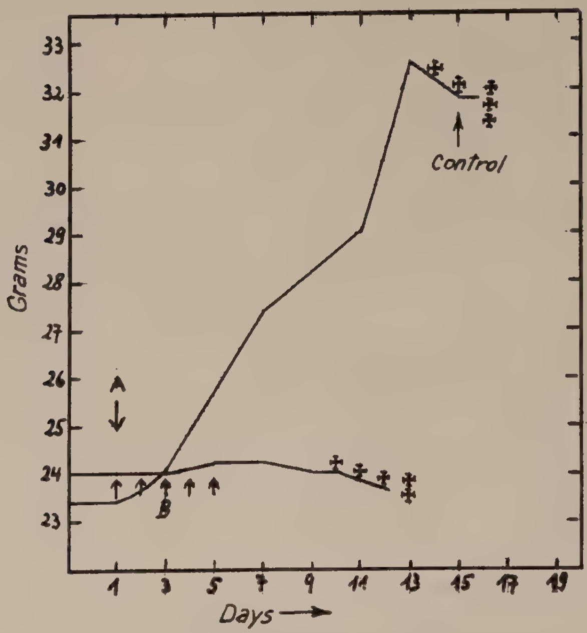

FIGURE 8. Example of a compound changing the weight curve, but without positive effect on the survival time. One mg. Diels' acid injected at *B*.

equality of the weight curves of both groups. In cases of an effective compound a further criterion is necessary, that is, the comparison of the survival times of the different groups. In the case of colchicine (FIGURE 7), we see that the treated animals live longer than the control animals; a quantitative calculation shows that the survival time is doubled.[3] The same results with colchicine with this same tumor strain have been described by Creech *et al.*,[11, 12] in contrast to the results of Sugiura.[12] In many cases we have observed a striking difference between the weight curves, but no difference between the survival times (FIGURE 8). Thus, this second criterion must be used for the evaluation of the effect of a compound.

Which differences of survival time are significant? The general deviations of survival times of a greater number of animals give the impression of a greater variability than is demonstrated by the single experiment. As a matter of personal judgment, some differences in the survival times are defined as ±, +, or ++. A quotient of 1.5 or more of the average of survival times of experimental and control groups is significant for a positive effect. One of the great advantages of this test method is the opportunity for studying the dosage dependence with respect to the effect of a compound. In TABLE 4 the effects of different dosages of colchicine are compared,[9] expressed by the quotient of the 50 per cent survival time (S_{50}) of the experimental and of the control group. With small doses we notice no effect; with increasing doses there is an increasing effect; then, again, a decrease of the effect is seen, caused by

TABLE 4

DOSAGE DEPENDENCE OF THE EFFECT OF COLCHICINE

Daily dosage (μg.)	I S_{50} Control	II S_{50} Experiment	II/I*
2.5	16.4	17.0	1.0
5.0	13.6	26.8	2.0
10.0	13.6	34.8	2.6
15.0	14.2	22.0	1.6
20.0	13.6	5.2	0.4
30.0	13.6	4.0	0.3

* II/I = quotient of S_{50} of the experimental group and of the S_{50} of the control group.

TABLE 5

DOSAGE DEPENDENCE OF THE EFFECT OF ACRIFLAVINE

Daily dosage (μg.)	I S_{50} Control	II S_{50} Experiment	II/I*
25	15.0	18.7	1.2
50	16.2	30.4	1.9
100	14.6	62.2	4.3
150	15.0	64.0	4.3
200	19.0	29.0	1.5

* II/I = quotient of S_{50} of the experimental group and of the S_{50} of the control group.

the toxicity of the compound. From the data presented in this table, I should like to emphasize the fact that there exists (1) an optimal dosage of activity and (2) a dosage with which the effect will again disappear. Both dosages seem to be good characteristics for a compound. The second value is of interest regarding the question as to whether the toxicity of a compound is the same for normal animals as for animals bearing tumor. In TABLE 5 the analogue dosage dependence is given for acriflavine.[13]

In the course of our experiments we have observed all possibilities of variations of the type of weight curves (see, for example, FIGURE 8). A special point is the possibility of accelerating the growth of this tumor. Occasionally we have seen such an increase of the weight curve, but for the most part the effects have not been reproducible. The only compound we found to be accelerant was thioctic acid (α-lipoic acid), which caused a rapid growth of this tumor and an earlier death of the treated animals. With α-lipoic acid, there was a daily weight increase of 2.3 gm., a value never found among untreated animals (compare with TABLE 1).

Another advantage of the ascites tumor is that it creates the possibility of morphologic study of the behavior of the cells with the compounds present. According to the method of Brodersen,[14] mice with a well-developed ascites tumor at an age of 5 to 8 days are used. By tapping the tumor, smear preparations are produced that can be stained and, by counting the mitotic figures of 1000 cells, the mitotic rate is ascertained. From time to time after the injec-

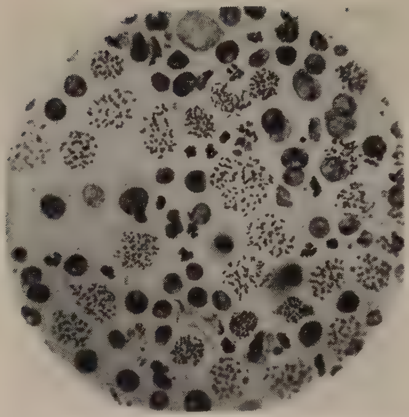

FIGURE 9. Accumulation of arrested metaphases 24 hours after the injection of 8 μg. of a colchicine derivative (NMC). Smear preparation; Feulgen stained. ×400.

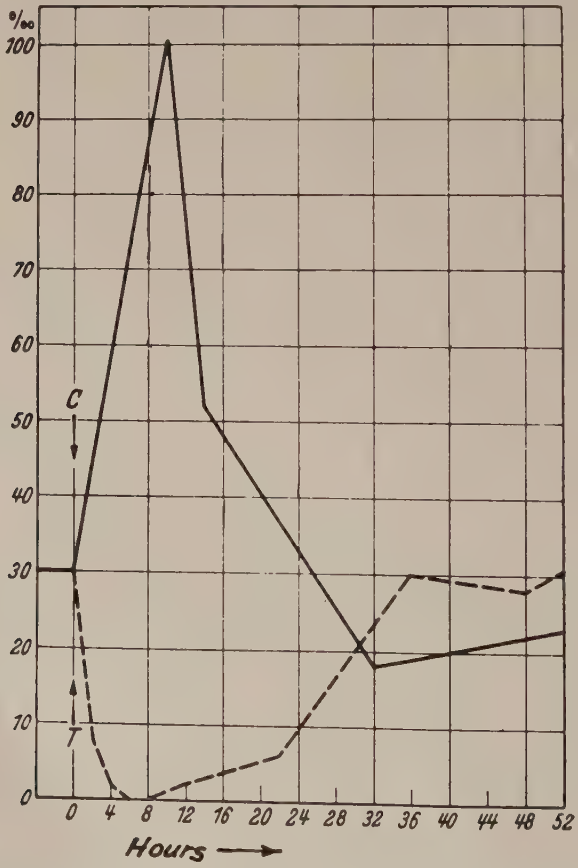

FIGURE 10. Mitotic rate (per thousand) in the ascites tumor. Continuous curve, 20-μg. injection of colchicine at *C*; dotted curve, 100-μg. injection of acriflavine at *T*.

tion of a compound, material can be taken out again, and the changes in these new preparations are followed. Using a mitotic poison, for instance, we observed an accumulation of arrested metaphases (FIGURE 9). The quantitative enumeration gave curves such as those shown in FIGURE 10.[14] Acriflavine, in contrast to colchicine, causes a decrease of the mitotic number (FIGURE 10). This method of arresting mitosis by colchicine derivatives gives both a great number of mitotic figures and figures of good cytological quality, so that this method has also been a basis for counting the chromosome number.[4, 18]

By this cytological method we have studied another question, perhaps of general interest in the field of chemotherapy of tumors.[15] Is the effect of a single injection of a large dosage of a compound equivalent to several injections of small doses fractionated over a longer time? In FIGURE 11 we see the result of a single dosage of 20 μg. *N*-methylcolchicamide (NMC) compared with the result of 2 dosages of 10 μg. and 6 dosages of 3 μg. We observe that the fractionation of the dosage has a greater effect than the single injection.[15] This means that it is possible to find an optimal mode of fractionation of a given amount of a compound and also an optimal sequence of injections, both being characteristic for the compound and the tumor used.

In experiments of this type we never succeeded in arresting 100 per cent of the tumor cells in mitosis. This led to the question of whether this is fundamentally impossible or whether there are cells less sensitive. We transplanted

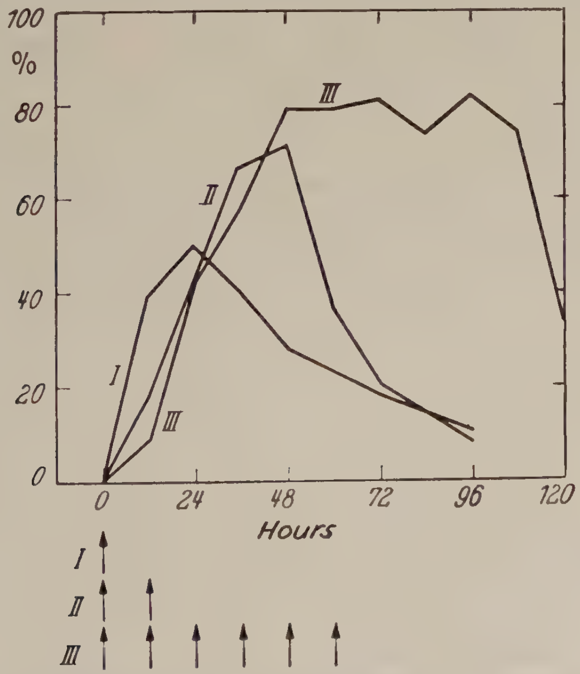

FIGURE 11. Effect of fractional application of NMC. The ordinate represents the percentage of arrested metaphases and polynuclear cells; curve I, 1 × 20 μg. NMC; curve II, 2 × 10 μg. NMC; curve III, 6 × 3 μg. NMC.

TABLE 6

COLCHICINE-RESISTANT SUBLINE OF THE EHRLICH ASCITES CARCINOMA

Year	Number of mice	S_{50} (days)*	μg. NMC
1952	237	10.5	5 × 3
1953	228	10.6	5 × 3
1954	171	11.9	5 × 1
1955	175	11.1	5 × 1
1956	246	10.8	3 × 1
1957	230	10.5	2 × 1
	1287	10.9	
1956	202	13.2	none
1957	248	13.4	none
		13.3	

* S_{50} = period in days during which 50 per cent of the animals died, dating from the years 1952 to 1957, with and without injection of NMC.

cells of the ascites tumor, pretreated with colchicine or NMC, and treated this tumor again during its growth in the animal. In most of these experiments the tumor died out during several transfers of this type. In one case we obtained a resistant strain,[16, 17] which has been maintained since 1951.

TABLE 6 shows the average survival times of animals with this tumor in the years 1952 to 1957. In comparison with the original strain, the survival time has decreased from 14.0 to 10.9 days. This strain was bred under injection of NMC starting with 3 μg. daily for 5 days. Later we were forced to reduce this amount, as shown in TABLE 6, because the higher dosage caused earlier death of the animals. In 1956 we branched off a line bred without injection of NMC. The average survival time of animals in this line is 13.2 days. There is some evidence that this tumor is not only resistant but also dependent on NMC. The subline divides by mitosis, and the cells have a cell spindle. The number of chromosomes was counted by Querner, and the results from the 287th transfer are given in TABLE 7 in comparison with the values for the original tumor, as found by Bayreuther,[4] and for a clone derived from the original tumor by single-cell transplantation.[18] This resistant subline proved also to be diploid.

As mentioned above, in 1941 I had calculated the doubling time of the colchicine-sensitive hyperdiploid Ehrlich ascites carcinoma to be 2 to 2.5 days. In the meantime Klein and Révész,[19] Patt and Straube,[20] and Lucké and Berwick[21] have made other calculations about the doubling time, starting from the assumption that the cube root from cell mass is proportional to time. Without discussing this question, I should like to mention another method for determining the doubling time. Wyckoff (Labaw *et al.*[22]) showed, with *Escherichia coli*, that cells labeled with radioactive phosphate do not lose phosphate during divisions, so that the total amount of activity of all cells is the same even if the cell number increases. The same effect has been demonstrated by Friedrich-Freksa and Kaudewitz[23] with *Amoeba proteus*. Naturally, the

TABLE 7

CHROMOSOME NUMBERS IN THE DIPLOID EHRLICH ASCITES CARCINOMA AND SOME SUBLINES*

Chromosome number	Colchicine		
	Sensitive strain		Resistant strain
	Original strain	Clone IX	
–30	0	1	2
31–40	0	29	48
41–50	93	20	39
51–60	0	0	5
61–	7	0	6
	100	50	100

* Taken from experiments by Bayreuther (original colchicine-sensitive strain),[4] by Querner (clone IX derived from the original strain), [18] and by Querner (colchicine-resistant subline) unpublished.

TABLE 8

APPARENT DOUBLING TIMES OF THE EHRLICH ASCITES TUMOR CELLS, CALCULATED FROM (1) THE INCREASE OF CELL NUMBER AND (2) THE DECREASE OF THE INITIAL ACTIVITY OF P^{32}-LABELED TUMOR CELLS

Age of the tumor (days)	Doubling time (days) calculated from:			
	cell number		dilution of activity	
7	1.6	1.9	1.2	1.3
9	1.8	2.0	1.3	1.5
10	2.3	2.8	1.7	1.7
11	2.0	2.4	1.5	1.8
12	2.4	2.7	1.5	2.0
14	3.3	3.4	2.5	2.6

activity per cell decreases, and we can make use of the dilution of radioactivity during tumor growth for an estimation of the doubling time.[24] While the cell number increases, the activity per cell decreases:

$$A_t = A_0 \cdot 2^{-t/t_M}$$

$$Z_t = Z_0 \cdot 2^{t/t_M}$$

Now we can find the doubling time t_M by (1) counting the cell number and (2) following the radioactivity of the cells.

In TABLE 8 are given the results obtained with tumors of different ages, ranging between 7 and 14 days.[24] From these values of doubling times we see, first, that those indicated by the "activity dilution method" are smaller than those obtained by cell counts. The method of counting cells has a greater probability for error, as it is practically impossible to observe all cells. Second, we see the dependence of doubling time on the age of the tumor, the t_M

TABLE 9

APPLICATION OF THE "ACTIVITY DILUTION METHOD" ON THE DETERMINATION OF THE APPARENT DOUBLING TIME OF TUMOR CELLS UNDER THE INFLUENCE OF DIFFERENT INHIBITORY AGENTS

Days after inoculation	Control	NMC		Acriflavine 60	Patuline 80
		3*	9		
14	2.3	4.0	⊖	⊖	⊖
19	+	+	5.6	5.6	5.5

* Dosage of the compounds in μg. daily for 5 days.
Symbols: ⊖ = not yet ascites formation; + = all animals dead.

values increasing with the age of the tumor. Therefore, these values are to be considered only as average values or, better, as values for an "apparent doubling time," ignoring all possible deviations from the formula used.

In the cases of NMC, acriflavine, and patuline, we have used this method to determine the apparent doubling time in the tumor with the presence of these compounds.[25] In TABLE 9 these values are presented, demonstrating that, instead of the value of 2.3 for the control group, we have in these cases values of 5.0 and more. I mention this method here because it represents another variation of the use of the ascites tumor in screening procedures. The method is simple; it requires only the measurement of the activity of the inoculated material at the time *t* of an uninfluenced tumor and of the treated tumor at the same time. As the activity can be measured with small samples, one would be able even to take sufficient material from the living animals. Thus, further continuation of the experiment would not be disturbed, nor would the important characteristic of the survival time of the animals be missed.

In uninfluenced growth during the development of the ascites tumor, the growth of solid tumors inside the body of the mouse is irrelevantly small within the period of the normal survival time. However, in animals with inhibited growth of the ascites tumor and with a longer survival time we can observe the development of solid tumors inside the animal. This is another phenomenon to be checked; in some cases the development of solid tumors is extremely large.

According to the title, this paper is concerned only with the action of mitotic poisons on this hyperdiploid Ehrlich mouse ascites carcinoma. Naturally, however, one can make use of this object in studying the effect of every other cytotoxic compound not acting as a mitotic poison.

This method has served us as a valuable tool in a great number of our experiments. The advantage of the method is that it requires only a relatively small number of animals and supplies a quick answer, especially in the case of negative compounds. When I worked out this method in 1940 I shared the prevalent belief that results with this one tumor would be valid for all other tumors. It is the general problem of present cancer research and of this monograph to find a screening procedure that really possesses this ideal quality. Among all the possibilities of known screening procedures, the use of the mouse ascites tumor is only one method, possessing some advantages and some limitations in the general field.

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PARAMETERS OF ASCITES TUMOR GROWTH*

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Ascites tumors, because of their analytical convenience and biological flexibility, represent an adaptation important for study of growth. Such tumors, like all transplantable tumors, may be subject to influences not operative in the development of naturally occurring neoplasms. The ascitic form of growth is nevertheless particularly useful in that patterns of cell proliferation can be studied directly under a variety of conditions and, by contrast with the corresponding solid growth, variables of organization and vascularization can be evaluated. Applications and advantages of ascites tumors for chemotherapeutic screening have been discussed by Hauschka and his associates[1] in a summary report to the Cancer Chemotherapy National Service Center, Bethesda, Md.

A screening procedure is, in essence, an exploratory study, although it has a somewhat greater background for discrimination. The ultimate objective of chemotherapeutic screening is, of course, tumor curability. This, in the strict sense, may be too confining for an initial screening procedure, and it is customary in the search for active agents to use some measure of tumor sensitivity instead. It will be remembered that sensitivity and curability may reflect different points of emphasis: for example, the probability of injury as opposed to the probability of recovery. Hence, a parameter of the one is not necessarily paralleled by the other.

Various parameters have been used for evaluation of inhibitory effects on ascites tumor growth. A distinct advantage of these tumors is the fact that growth measurements may be made directly by determining the total number of free tumor cells in the peritoneal cavity. The extent to which such quantitation is possible will depend upon the variety of ascites tumor, the size of the inoculum, and the time of sampling in relation to the chronology of tumor development. As discussed elsewhere,[2] factors of latency, invasiveness, and cell aggregation may complicate interpretation of the growth curves.

Several procedures, for example quantitative recovery of peritoneal contents or dye or isotope dilution in conjunction with cell counts, have been used for enumeration of the total free tumor cell population. Although one or another may be more appropriate under different circumstances, the methods, in general, correspond closely. In many instances tumor development can be approximated by the mean total cell mass calculated from the percentage of packed cells and the total ascites weight. The former is determined by centrifugation, the latter by weighing the animal before and after careful peritoneal drainage. The mean total cell mass is a convenient parameter for evaluation of drug effects on tumor growth. It is limited by possible changes in mean tumor cell volume as a result of treatment. Thus, inhibition of cell division but not of cell growth, which may occur, for example, after appropriate irradiation, may not be readily detected by this procedure.

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Results obtained with several mouse ascites tumors reveal that tumor development consists of an initial phase of rapid cell multiplication followed by a period of decelerated growth toward an asymptote. There is a variable delay in the onset of growth after tumor inoculation. The latent period apparently depends upon the inoculum, the tumor, and the host. There may be little, if any, lag under some conditions, and it is of interest that the presence or absence of latency seems to be related to the age of the host under otherwise comparable conditions (R. L. Straube,, unpublished data). In most instances there is an exponential increase in the number of tumor cells during the first few days of active growth. The over-all pattern for a given ascites tumor appears to be a function mainly of the total number of free tumor cells.[3] However, the principal determinant of growth limitation is the total neoplastic mass rather than cell number per se.[4] It is noteworthy that Ehrlich tumor cells can be maintained in a log phase of growth by repeated removal of ascites (R. L. Straube, unpublished data). The animals succumb when the total neoplastic mass produced is comparable to that in an untapped mouse. Since the phase of rapid tumor growth is predictable and relatively uncomplicated, measurements should be made during this period for evaluation of possible chemotherapeutic effects.

It is important to emphasize that parameters such as survival time, body weight, tumor cell concentration, and mitotic index do not necessarily reflect the course of cell multiplication.[2] Survival free of tumor is the ultimate parameter for curability. Survival time is a reasonable criterion for primary screening, particularly when tumors with a narrow range of survival are tested. Differences in survival time, however, may be considerably in excess of the time required for attainment of equivalent cell numbers. Measurement of body weight may be misleading, since it is a complex function of tumor growth and, moreover, alteration in ascitic fluid volume may occur without comparable change in the tumor cell population. Cell concentration or packed cell volume per se has little meaning without the corresponding ascitic volume.

Frequent reference is made to the mitotic index as a growth parameter, and t is well to consider briefly its significance and limitations. The percentage of cells in division, or mitotic index, is related directly to the mitotic time and he skill of the observer and, inversely, to the intermitotic time. Since the mitotic index is usually computed in terms of the entire cell population, it does not provide a true indication of mitotic activity unless one is dealing with a homogeneous population in which all cells participate equally in mitosis. n most instances the generative cells represent only a part of the total, and his may also apply, to some extent, to populations of ascites tumor cells. Heterogeneity of the population does not preclude estimation of cell turnover n terms of the conventional mitotic index if the duration of mitosis is known nd an assumption is made concerning the linearity of the increase in number f cells. On the other hand, if an agent has a selective action that alters the istribution of generative and nongenerative cells, the significance of a change n the mitotic index expressed for the total population would be less obvious. f the proportion of proliferating to nonproliferating cells could be established, t would be possible to calculate the intermitotic time as well as the turnover time. The former is a more relevant parameter of sensitivity, for example, in evaluation of mitotic blocking agents.

Many ascites tumors of mice, believed to represent nearly pure cultures, in fact consist of heterogeneous cell populations varying in ploidy, size, and rates of proliferation.[5] Such heterogeneities may result in a wide spectrum of sensitivities; effects on more sensitive components may stimulate activity in the more resistant components in a manner analogous to the stimulating effect of heavily irradiated cells on the growth of unirradiated cells.[6-8] Considerations of this sort raise the question of a critical cell number for restoration of tumor growth in either the ascitic or solid form. This may be vastly different for transplantable and naturally occurring neoplasms. Since even immunologically nonspecific ascites tumors adjust to the host in which they are maintained, screening must be carried out without departures from the optimal strain. Although, superficially, ascites tumors would appear to represent a simple system for chemotherapeutic screening, as in the case of solid tumors, there are many uncertainties concerning the relationships between tumor growth and host response.

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RELATIVE SENSITIVITY OF THE SOLID AND ASCITES FORMS OF SARCOMA 180 AND EHRLICH CARCINOMA TO INHIBITORY COMPOUNDS*

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In recent years investigators have shown much interest in the use of ascites tumors in experimental cancer research, especially in the cytology and biochemistry of tumor cells. Ascites tumors also have been used to a considerable extent by investigators in chemotherapy studies. These tumors provide an easy challenge to chemotherapeutic agents because the intraperitoneal injection of compounds against the intraperitoneal ascites form of tumor is, in effect, an *in vivo-in vitro* test. Thus it is possible to obtain an idea of the action of a compound without as great a degree of intervention by various organs.

Three years ago in another publication of The New York Academy of Sciences I reported results obtained on comparing carcinostatic effects of 54 compounds on both ascites and solid forms of the same tumors.[1] The present report describes the testing of 122 selected compounds on Sarcoma 180 and the Ehrlich arcinoma, both solid and ascites forms.

Materials and Methods

Upon intraperitoneal injection of fresh ascites fluid containing about 1 million cancer cells (FIGURE 1), mice regularly develop large amounts of milky scites (5 to 20 cc.) in 7 to 14 days and die in 10 to 20 days (FIGURE 2). The umors have 100 per cent "takes" and, generally, no spontaneous regressions.

The milky fluid of Sarcoma 180 ascites tumor contains about 150 million umor cells/cc., while Ehrlich ascites carcinoma contains about 100 million ancer cells/cc. By differential fluorochroming ascites tumor cells after staining with acridine orange, however, it was estimated that about 4 per ent of these tumor cells are dead.[2] Dead cells have red cytoplasm.

There are several ways of determining the effectiveness of a compound gainst the ascites form of tumors: (1) cytological examination of ascites umor cells from treated animals;[3, 4] (2) counting the number of tumor cells t various time intervals in ascitic fluid after treatment;[5] (3) determination f the cell volume obtained by means of centrifugation of aliquots of ascitic luid (total tumor packed cell volume);[6] (4) determination of survival time n treated animals;[4, 5, 7-9] (5) measurement of the amount of ascites formed fter treatment (by visual estimation of the abdominal distention, by weighng the mice, or by withdrawing ascitic fluid with a syringe and measuring ts volume);[10] and (6) a dye-dilution technique to estimate ascitic-fluid volume.[11-13]

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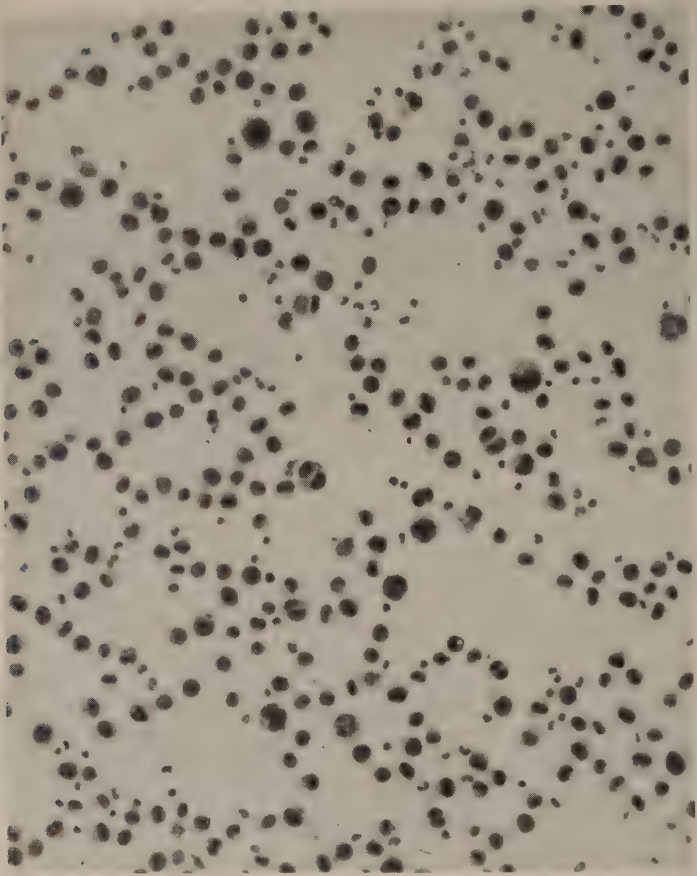


FIGURE 1. General view of the smear of Ehrlich ascites tumor, showing an abundance of active cancer cells (large cells) and a small number of normal cells (small cells). Papanicolaou stain. ×200.

Chemotherapy test. We adopted the fifth procedure for chemotherapeutic studies with ascites tumors. A donor mouse was selected among mice having rapidly accumulating ascites (moderate to marked abdominal distention) at 7 to 10 days after inoculation of cancer cells. One to 3 cc. of milky or slightly hemorrhagic ascitic fluid was withdrawn with a syringe fitted with a 20-gauge needle. The cells were then counted in a hemocytometer, and a proper dilution was made with physiological salt solution at *p*H 7.0. Intraperitoneal injection of 0.1 cc. of the fluid, containing about 1 million cancer cells, into young healthy mice (Rockland Swiss albino, weighing 18 to 22 gm.) was made in the inguinal region with a $\frac{1}{4}$-cc. syringe having a 25-gauge needle. Aseptic precautions were taken during the procedure. The animals were maintained on a standard pellet diet (Purina Laboratory Chow) and water.

For the solid tumors, subcutaneous implantations of small pieces of tumor,

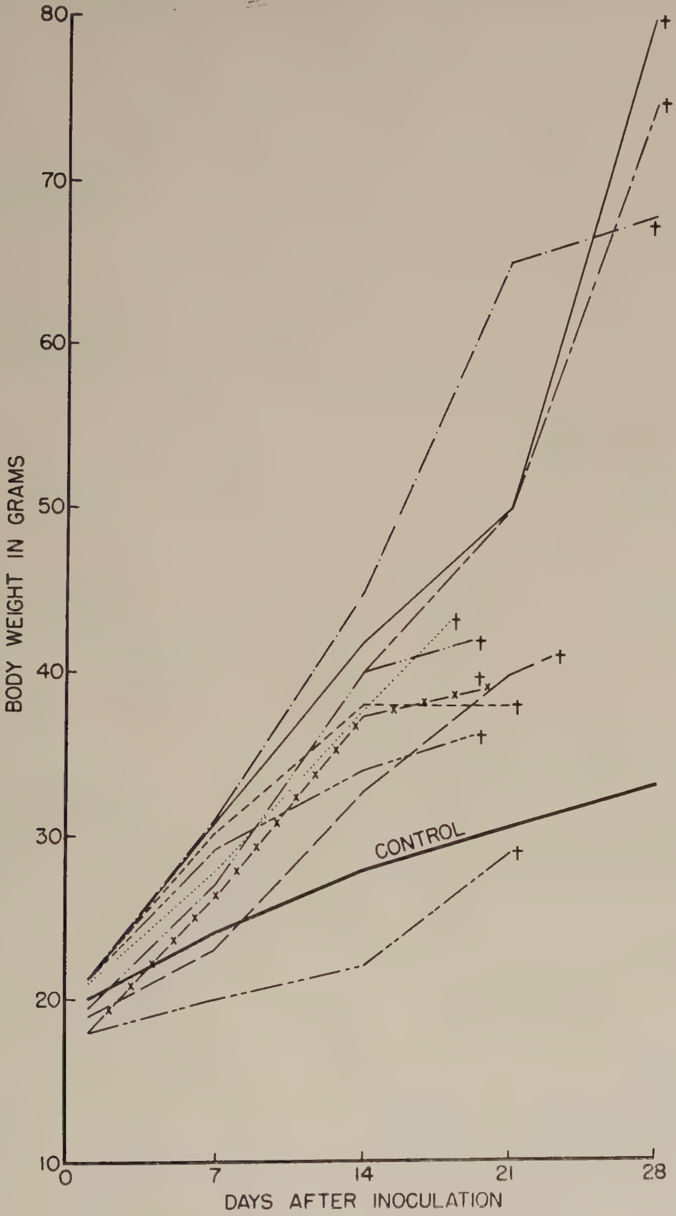


Figure 2. Weight curves of mice after injection of 1 million Ehrlich ascites tumor cells. The heavy, continuous line (control) indicates the average growth curve of 30 normal young male mice. Each of the other lines indicates the growth of one ascites-injected young male mouse. Death is indicated by a cross (†).

TABLE 1

EFFECT OF VARIOUS COMPOUNDS ON SOLID AND ASCITES FORMS OF THE SAME TUMOR

Compound	Solvent	Dose mg./kg./day	Sarcoma 180		Ehrlich carcinoma	
			Solid	Ascitic	Solid	Ascitic
Folic acid	S	50	−	−	−	−
A-Methopterin	S	1.0	+	++	−	±
Colchicine	S	0.5	−	+++	−	+++
N-Desacetylthiocolchicine	S	80	−	+	−	+
Urethan	S	500	+	±	−	+
Carbamic acid, methyl ester	S	500	−	−	−	−
N-Methylformamide	S	250	±	−	+	±
N-Methylacetamide	PG	1500	−	−	±	−
HN2	S	0.5	±	+++	±	+++
Triethylene melamine	S	0.5	+	++	±	+++
Triethylene phosphoramide	S	6.0	+	±	+	++
p-Phenylenediphosphoric acid tetraethylenimine	S	40	±	+++	+	+++
Myleran	PO	20	−	++	±	−
1,9-Di(methanesulfonoxy)nonane	PO	50	−	±	±	+
Pyrimidine	S	500	−	−	−	−
2,4-Diamino-5,6-dimethylpyrimidine	CMC	50	−	−	±	−
2,4-Bis(ethylenimino)-6-chloropyrimidine	S	2.0	++	+++	−	+++
5,7-Diamino-1-v-triazolo-(*d*)-pyrimidine	CMC	100	±	−	+	−
5,7-Dihydroxy-1-v-triazolo-(*d*)-pyrimidine	CMC	300	−	−	−	−
5-Hydroxy-7-amino-1-v-triazolo-(*d*)-pyrimidine	CMC	25	±	−	−	−
4-Methylaminopyrazolo-(3,4-*d*)pyrimidine	CMC	7.5	−	−	−	−
4-Aminopyrazolo-(3,4-*d*)pyrimidine	CMC	10	−	−	±	±
3-Allyl-5-cyanocytosine	CMC	500	−	−	−	−
Purine hydrochloride	S	250	±	−	±	−
6-Mercaptopurine	CMC	30	+++	+++	+	++
6-Mercaptopurine riboside	CMC	125	±	−	±	−
6-Mercaptopurine glucoside	S	250	+	+	±	+
2,6-Diaminopurine	S	60	−	−	±	−
Thioguanine	CMC	3.0	+	+++	+	+++
Thioguanine riboside	CMC	2.5	±	+	±	+
6-Methylmercaptopurine dihydrate	CMC	50	−	−	−	−
6-α-Furfurylamino-9-β-D-ribofuranosyl purine	CMC	250	−	+++	±	+++
Purinyl-6-thiocyanate	CMC	10	±	−	±	−
Purinyl-6-disulfide	CMC	20	+	++	+	+
5-Fluorouracil	S	25	+	+++	±	+++
5-Fluoroorotic acid	S	20	±	+++	−	+++
5-Bromouracil	CMC	500	−	−	−	−
Uracil-5-carboxylic acid	S	125	±	−	+	−
5-Fluorocytidine	S	10	−	+++	±	++
5-Fluorouridine	S	5.0	−	+++	±	+++
5-Fluorodeoxyuridine	S	40	++	+++	−	+++
5-Hydroxyuridine	S	400	±	±	±	++
6-Azathymine	S	500	−	−	−	−

TABLE 1—*Continued*

Compound	Solvent	Dose mg./kg./day	Sarcoma 180		Ehrlich carcinoma	
			Solid	Ascitic	Solid	Ascitic
Cortisone acetate	S	37.5	−	+	±	++
Hydrocortisone acetate	CMC	37.5	±	++	+	++
1-Dehydro-9-α-fluorohydrocortisone acetate	CMC	12.5	−	−	−	+++
9-α-Fluoro-2-α-methylhydrocortisone acetate	CMC	25	±	++	±	+
Testosterone propionate	PO	500	−	−	−	−
17-Ethynyltestosterone	CMC	375	−	±	−	−
19-Nortestosterone	CMC	500	−	−	−	±
17-Ethynyl-19-nortestosterone	CMC	500	−	−	±	−
Pregnenolone	CMC	100	−	−	−	−
Estradiol	CMC	40	−	−	±	±
Diethylstilbestrol	CMC	10	−	±	−	−
Ethionine	CMC	250	−	−	−	−
Azaserine	S	5.0	++	+	±	+
DL-Serine, *O*-methanesulfonate ester	S	50	−	−	−	−
6-Diazo-5-oxo-L-norleucine	S	0.1	++	++	+	++
DL-Hydroxynorleucine	S	500	−	−	−	−
L-Glutamic acid, γ-hydrazide	S	125	−	+	−	++
3,3-Dimethyl-1-phenyltriazene	PO	50	+	++	++	++
3,3-Diethyl-1-phenyltriazene	PO	35	−	+	−	±
3,3-Dimethyl-1-*p*-tolyltriazene	CMC	150	+	+++	+	+++
3,3-Dimethyl-1-*p*-nitrophenyltriazene	CMC	250	±	+++	+	++
3-Cyclohexyl-3-methyl-1-*p*-tolyltriazene	PO	250	−	−	−	−
3,5-Dihydroxy-1,2,4-triazine	CMC	500	±	−	+	−
Quinoline	PG	75	−	−	−	−
Quinoline-*N*-oxide	S	250	±	−	+	−
4-Nitroquinoline-*N*-oxide	CMC	10	−	+++	+	+++
6-Nitroquinoline	CMC	75	−	+	−	++
8-Nitroquinoline	CMC	62.5	−	±	±	−
2-Ethyl-4-nitroquinoline-*N*-oxide	CMC	8	−	+++	±	+++
4-(*p*-Nitrostyryl)quinoline	CMC	250	−	+	−	−
4-Carboxaldehyde thiosemicarbazone quinoline	CMC	100	−	±	−	−
8-Nitro-1-methyl-2-quinolone	CMC	125	−	−	−	+
Penicillin	S	2000	−	−	−	−
Streptomycin	S	500	−	−	−	−
Chlortetracycline	S	50	−	−	−	±
Illudin M	S	0.5	−	−	±	−
Gramicidin	CMC	12.5	±	−	−	+
Acti-dione	S	25	−	±	−	−
Puromycin	S	100	−	−	±	−
Fumagillin	CMC	10	+	+	+	++
Actinomycin C	A and S	0.025	−	+++	±	+++
Actinomycin D	A and S	0.025	−	+++	−	+++
Actinomycin J	A and S	0.025	−	+++	±	+++
Sarkomycin	S	250	−	+++	±	+++
Carzinophilin	S	2500 units	−	+++	−	+++
Pentamycin	S	0.5	−	−	−	−
Kanamycin	S	500	−	−	−	−
Mitomycin C	S	2.0	+	+++	±	+++
Benzimidazole	S	250	−	−	−	−
2-Ethylamino-1,3,4-thiadiazole·HCl	S	75	−	−	−	±
5-*tert.*-Butylbenzimidazolone	CMC	125	−	−	−	−
2-Allylimino-1,3,4-thiadiazoline·HCl	S	100	±	−	±	+

TABLE 1—*Continued*

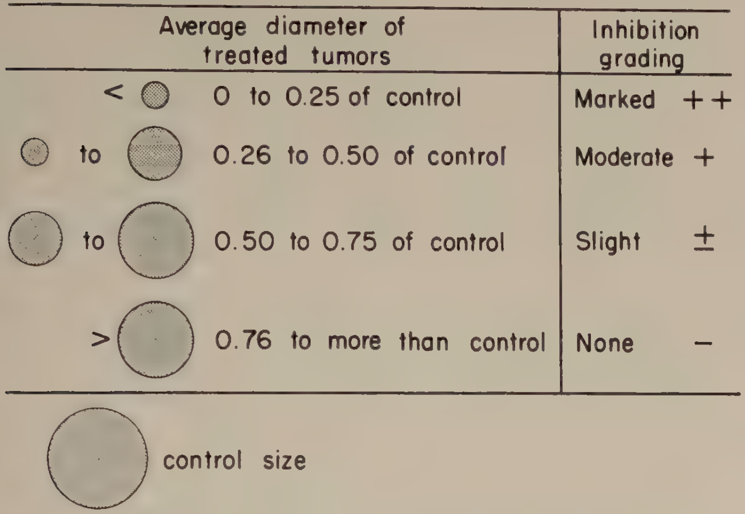
Compound	Solvent	Dose mg./kg./day	Sarcoma 180		Ehrlich carcinoma	
			Solid	Ascitic	Solid	Ascitic
l-Parsidol	CMC	100	–	–	–	–
d-Parsidol	CMC	100	±	±	–	–
p-Diphenylamine diazonium bromide	S	2.0	–	±	–	–
p-Diphenylamine diazonium fluoroborate	S	0.1	–	–	–	–
2-Amino-5-nitrothiazole	CMC	50	–	–	±	–
Quinine	CMC	125	–	±	±	–
Aspirin	S	150	–	+++	–	++
Reserpine	S	0.5	–	–	–	+++
6-Aminonicotinamide	CMC	5.0	–	–	±	±
p-(Diethyltriazeno)benzenesulfonamide	CMC	175	–	+	±	+
N-(*p*-Fluorophenacyl)-2-bromopyridinium bromide	S	10	–	–	–	–
Ethylenediamine tetraacetic acid	CMC	125	–	–	–	–
Diaziridinylphosphonitrile	S	0.25	–	–	–	–
Lutrexin	CMC	500	–	–	–	–
5-Fluoroanthranilic acid	CMC	75	–	–	–	–
Methylene blue	S	30	–	–	–	–
Alazopeptin	S	0.05	+	±	±	–
o-Phenylenediamine	S	200	–	±	++	+++
Galactose	S	5000	–	±	±	–
Paraformaldehyde	S	125	–	+	–	+
Propylene glycol	PG	250	–	–	–	–
α-Ethylbenzyl alcohol	PO	10	–	–	±	–
Cetyl alcohol	CMC	500	–	+	–	±
4-Desoxypyridoxine phosphoric acid	CMC	250	–	–	–	±
H_2O_2	S	150	–	–	–	–
Potassium arsenite	S	10	–	–	–	–

each weighing approximately 6 mg., into healthy young mice were carried out by the usual trocar method with a single implant into the right axillary region. In every set of experiments, tumor-bearing animals were divided into 2 groups, one of controls and the other for treatment with chemotherapeutic agents. The progress of the tumors in the animals was recorded graphically by measuring them in 2 diameters with calipers at weekly intervals for 4 weeks after tumor transplantation.

The first intraperitoneal or subcutaneous injections of compounds at maximum tolerated doses were given 1 day after inoculation of tumor fragments or intraperitoneal injection of 0.1 cc. of the fluid containing about 1 million cancer cells; the treatment was continued for 7 days.

The solvents used to dissolve the compounds and antibiotics are listed in TABLE 1. The injection volumes of the solvents used for the chemotherapeutic agents were as follows: saline (S), 0.5 cc.; carboxymethyl cellulose (CMC), 0.5 cc.; alcohol and saline (A and S), 0.5 cc.; peanut oil (PO), 0.1 cc. In all cases, injections were made once daily. Dosages of 0.1 to 0.2 cc. were used for all subcutaneous treatments.

The degree of inhibition of growth of the solid tumors was graded according to the following scheme (FIGURE 3): +++, complete destruction of tumors (complete regression of more than 50 per cent of tumors in the treated animals); ++, marked inhibition (failure to grow, or growth to approximately one



Average diameter of treated tumors		Inhibition grading	
< ●	0 to 0.25 of control	Marked	++
● to ●	0.26 to 0.50 of control	Moderate	+
○ to ○	0.50 to 0.75 of control	Slight	±
> ○	0.76 to more than control	None	−

○ control size

FIGURE 3. Degree of inhibition of solid tumor growth.

fourth of the average diameter of the controls); +, moderate inhibition (tumor growth from one fourth to one half of the diameter of the controls); ±, slight inhibition (tumor growth from one half to three fourths of the diameter of the controls); and −, no effect (tumor growth to three fourths or more of the diameter of the controls).

The degree of inhibition of ascites tumor growth was graded according to the following scheme (FIGURE 4): −, no effect, indicating marked abdominal

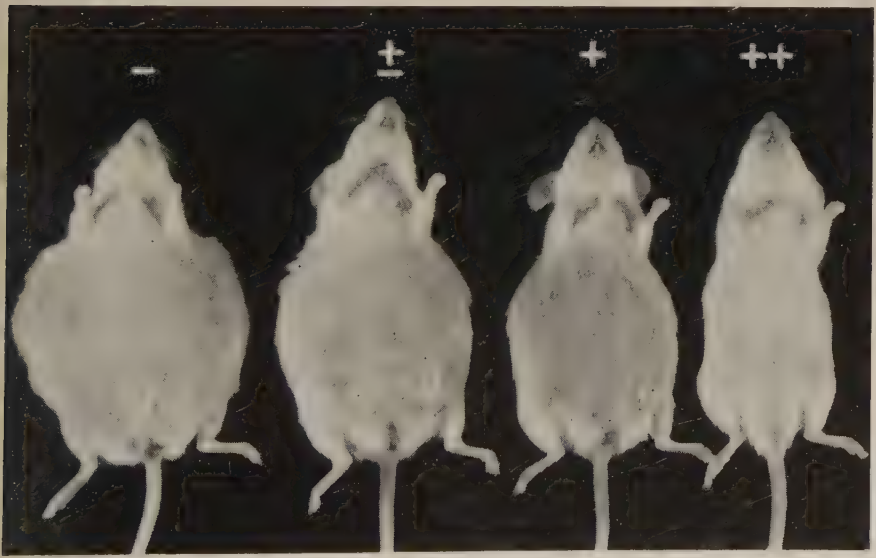


FIGURE 4. Degree of inhibition of ascites tumor growth: − indicates no effect; ±, slight inhibition; +, moderate inhibition; ++, marked inhibition.

distention, with the fluid volume increasing to three fourths or more of the controls (10 to 25 cc. ascites); ±, slight inhibition, indicating moderate abdominal distention, with the fluid volume increasing to about one half the volume of the controls (5 to 9 cc. ascites); +, moderate inhibition, indicating slight abdominal distention, with the fluid volume increasing to about one fourth the volume of the controls (1 to 4 cc. ascites); ++, marked inhibition, indicating no abdominal distention (no gross ascites); and +++, indicating complete inhibition or destruction of ascites.

Results

Results obtained with the 122 selected compounds on the 2 mouse tumors' both solid and ascitic forms, are shown in TABLE 1. The compounds investigated have been folic acid analogues, carbamates, formamides, nitrogen mustards, phosphoramides, pyrimidines, purines, steroids, amino acids, triazenes, quinolines, antibiotics, inorganic salts, and other compounds of chemotherapeutic interest. Each compound was tested, using 5 to 10 mice bearing the solid or ascites tumors; in most cases, experiments were repeated. Several of the representative compounds previously reported[1] were included in TABLE 1.

It is apparent from the results that, in 67 per cent of the cases, the responses to agents were identical or almost identical in both ascites tumors. In the remaining 33 per cent the responses were of different magnitude. 5-Hydroxyuridine had a marked inhibitory effect on Ehrlich ascites carcinoma, but it had only a slight inhibitory effect on Sarcoma 180 ascites tumor. *o*-Phenylenediamine and reserpine had a destructive effect on Ehrlich ascites tumor, but it had only a slight or no inhibitory effect on Sarcoma 180 ascites tumor. In the case of Myleran, there was actually a difference in results, for there was an inhibitory effect on Sarcoma 180 ascites tumor, but it had no effect on Ehrlich ascites carcinoma.

Subcutaneous injection of compounds tested, except cortisone and other steroids, was less effective than intraperitoneal injection. Cortisone and hydrocortisone, given subcutaneously, caused a marked inhibitory effect on both ascites tumors, but testosterone and pregnenolone, given subcutaneously, had no inhibitory effect. Repeated intraperitoneal injections of 37.5 mg./kg. of cortisone or hydrocortisone had only a slight inhibitory effect on ascites tumors.

It was of interest to note that, when the responses of the solid tumors and ascites tumors to the 122 compounds were matched, they were found to agree in only 56 of 122 instances (46 per cent) in the case of Sarcoma 180, and 46 of 122 instances (38 per cent) in the case of Ehrlich carcinoma.

In practically all cases the ascites tumors were more sensitive to the carcinostatic action of agents than their respective solid tumors (examples: colchicine, HN2, 5-fluorouracil, hydrocortisone, 4-nitroquinoline-*N*-oxide, actinomycin D, aspirin). In several cases, solid tumors responded better than their respective ascites tumors, for example, Sarcoma 180 treated with urethan, azaserine, and alazopeptin, and Ehrlich carcinoma treated with *N*-methylformamide, 5,7-diamino-1-v-triazolo-(*d*)-pyrimidine, uracil-5-carboxylic acid, 3,5-dihydroxy-1,2,4-triazine, and quinoline-*N*-oxide.

In general, the compounds without effect on solid tumors also were negative on their respective ascites tumors (examples: folic acid, pyrimidine, 5-bromouracil, testosterone, ethionine, penicillin, 5-fluoroanthranilic acid, methylene blue, H_2O_2). No compounds without effect on ascites tumors caused marked inhibition of solid tumors. Of 122 compounds tested on both ascites tumors, 61, (50 per cent) were inactive on Sarcoma 180, and 61 (50 per cent) were inactive on Ehrlich carcinoma.

As in the case of solid tumors, injection of active compounds 5 to 7 days after inoculation of the ascites tumors was much less effective than administration starting 1 day after inoculation.

Many antibiotics were active against the ascites tumors. Such data lend support to the belief that an ascites tumor, possibly the Ehrlich carcinoma, might be useful as an initial screen for detecting activity, but the greater sensitivity of this tumor must be appreciated to understand the possible detection of excessive "false positives." In Japan the ascites tumors have been used extensively; sarkomycin, carzinophilin, actinomycin J, and mitomycin C were detected in this system. However, sarkomycin, carzinophilin, and actinomycin J had practically no inhibitory effect on all 16 solid mouse tumors (Sarcoma 180, Sarcoma T 241, Sarcoma MA 387, Ehrlich carcinoma, Bashford carcinoma 63, Adenocarcinoma E 0771, Miyono adenocarcinoma, Carcinoma 1025, Lewis bladder carcinoma, Lewis lung carcinoma, Wagner osteogenic sarcoma, Ridgway osteogenic sarcoma, Mecca lymphosarcoma, Gardner lymphosarcoma, Harding-Passey melanoma, and Glioma 26) tested except on Carcinoma 1025, Ridgway osteogenic sarcoma, and Harding-Passey melanoma. On the other hand, a new antibiotic, mitomycin C, had marked inhibitory effects not only on ascites tumors but also on 11 of 16 solid mouse tumors.[14] Such striking differences in response of various tumors against antibiotics emphasize the importance of the use of a spectrum of tumors for the chemotherapy screening program.

Summary

Quantitative evaluation of results in chemotherapy studies with ascites tumors has been placed upon as definite a basis as similar investigations with solid tumors. However, the formation of solid tumors at the site of injection along with the ascites may complicate the evaluation, whether it is based upon weight of the mouse or upon increase in survival time. It has been suggested[5] that treatment with chemicals may decrease the amount of ascitic fluid without significantly influencing the number of tumor cells. In such cases, misleading information would be obtained from measurements of volume or weight. This possible source of error, however, can be detected by total cell counts.

Comparison of carcinostatic effects of 122 compounds on both ascites and solid forms of the same mouse tumors has demonstrated that the ascites forms appear to be a more sensitive system for the detection of antitumor activity. Intraperitoneal injection of chemicals into ascites tumor-bearing mice, however, represents a form of intratumoral treatment and, as such, has possibly less significance than treatment of solid tumors growing at a distance from the injection site.

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EVALUATION OF THE PERFORMANCE OF INDICES OF TUMOR GROWTH IN THE TESTING OF TUMOR GROWTH INHIBITORS AGAINST THE NELSON ASCITES TUMOR

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The special nature of cancer chemotherapeutic screening presents a host of difficult methodological questions for which we lack many answers. Answers to similar questions derived from previous pharmacological or statistical theory and practice do not apply to cancer chemotherapy, or apply only with considerable modification.

The first difficulty is connected with the absence of chemotherapeutic agents known to be effective against solid tumors in human patients. In the traditional pharmacological bioassay situation there were, at the beginning, agents (such as quinine, cocaine, and morphine) known to be clinically effective in humans. The compounds to be tested were compared with these known standards and it was relatively simple to ascertain whether the design of the bioassay procedure worked. The cancer chemotherapy screening program can employ this approach to evaluation only to a limited extent. It is compelled chiefly to compare the results obtained with one animal tumor system with another such system rather than with clinical results.

The second problem arises from the fact that results obtained with different animal tumor systems tend to disagree with each other. Consequently the changing of the tumor or the changing of the host (or both) is likely to make some chemicals look more promising and other chemicals look less so. The only answer to this difficulty is the use of a spectrum of tumors for screening, since it cannot be expected that a single tumor would be able to select all useful agents.[1]

Within a single animal tumor system we often encounter the third type of difficulty. The results of cancer chemotherapy screening tests are highly sensitive to numerous details of the experimental protocol (such as, source of animals, their nutritional status, latent and manifest infection, technique of tumor transplantation, methods of administration of test compounds, and time and methods of measuring the tumor growth indices). The animal tumor system is a more delicate screening tool than the animal alone (as used, for example, in toxicity bioassay experiments) or even the animal germ systems used in the bioassay of antimicrobial agents. The growth of a transplanted tumor homograft is subject to a variety of immunogenetic factors, and calls for a high degree of technical skill, especially when the cancer chemotherapy screening program is operated on a large scale. The result of all these difficulties is that the measure of precision often employed may be insufficient for proper evaluation of screening results.

Finally, there is no generally accepted procedure for reporting results that would permit comparison between different indices. For example, a percentage of inhibition of tumor growth calculated on the basis of tumor diameters (linear dimension) would differ from one derived from a comparison of tumor volume or weight (cubic dimension). It would be relevant also whether we use the difference in, for example, tumor weights, their ratio, or some other function as a criterion of retardation of tumor growth induced by chemical treatment.

In the present report the data presented were collected in the course of an experiment specifically designed for the comparison of a series of indices of inhibition of tumor growth in order to obtain more precise information on their performance in cancer chemotherapy screening.

Methods and Procedures

ICR male mice, weighing 18 to 22 gm. at the time of the tumor inoculation, were inoculated intraperitoneally or intramuscularly with a suspension of the Nelson ascites mouse tumor.[2] The tumor inocula were prepared by pooling the ascites fluid from several donor mice and adjusting the cell count by dilution with the Locke-Ringer solution to a value of 100×10^6 cells per ml. The standardized inoculum contained 10×10^6 tumor cells in 0.2 ml.

Each individual experiment included 2 control groups of tumor mice. One, the "negative" control, was left untreated; the other, the "positive" control, was treated with 7 daily intraperitoneal injections (15 mg./kg./day) of 6-mercaptopurine (6-MP), starting on the day after tumor inoculation. The 6-MP was administered as a suspension in a 5 per cent solution of carboxymethyl-cellulose. The choice of 6-MP as the positive control was based partly on the fact that at this dose level it had been found previously to increase (by approximately 50 per cent) the median survival time of mice bearing the Nelson ascites tumor. We wanted to detect agents whose potency was this good, or better.

In addition to these two control groups of mice, each experiment included four groups of mice treated with various chemicals. Since the purpose of this presentation is to discuss the comparative performance of various tumor growth indices, only the results obtained with "negative" and "positive" (6-MP) controls will be reported.

Each group of animals in a given experiment consisted of 22 mice used as follows: 17 received intraperitoneal injections of a standardized inoculum of the Nelson ascites tumor; 10 of these were observed for 6 weeks. In addition, the total body weight change (carcass and ascites) was measured on the seventh and fourteenth days after tumor inoculation. Four other mice inoculated intraperitoneally were sacrificed on the seventh day, and the carcass weight change and total packed cell volume were determined (TPCV = ascites volume in a mouse × percentage of packed cell measured in a cytocrit).[3]

In the third group of 3 mice inoculated intraperitoneally, the number of survivors 20 days after tumor inoculation was determined. The rationale for this qualitative index and the results obtained are discussed briefly in Appendix 1 of this paper.

Five more mice were inoculated in the thigh muscles of the right leg with 10 × 10^6 tumor cells. Tumor diameters were measured with calipers in two dimensions on living animals, and the total body weight change (carcass and tumor) was determined on the seventh and fourteenth days.

Data for the 7 tumor growth indices were obtained in 12 individual experiments extending over a period of about 70 days; both the values for each individual animal and the calculated average values were subjected to statistical analysis.

Results

Although there are some irregularities due to deaths of animals, the design of the entire experiment retains a balanced character that facilitates statistical analysis (TABLE 1). The basic individual data are presented in TABLES 2 through 7, and the mean values for the six quantitative indices for the untreated and 6-MP series are tabulated in TABLE 8. This information represents the raw material for the evaluation of the indices.

Generally speaking, there are three broad criteria for indices. An index should be stable, that is, in the hands of different investigators or in repeated experiments by the same investigator it should give reasonably consistent results. An index also should be sensitive, that is, it should show a marked response when an effective agent is tested. Finally, an index should be economically convenient in terms of cost of animals and overhead expenses.

Considering only the criteria of stability and sensitivity of an index, it is usually necessary to consider them simultaneously because they tend to be inversely related, that is, as an index becomes more sensitive it is likely to be less stable. For this purpose some kind of sensitivity/stability ratio can be

TABLE 1
NUMBER OF OBSERVATIONS: SIX INDICES

Experiment No.	Survival time (1)		Body wt. change—car. & tumor (2)		TPCV (3)		Carcass wt. change (4)		Tumor diameter (5)		Total body wt. change—car. & solid tumor (6)	
	T	C	T	C	T	C	T	C	T	C	T	C
1	10	10	10	5	3	3	3	3	5	5	5	5
2	10	10	10	8	4	2	4	2	5	5	5	5
3	10	10	10	9	4	4	4	4	5	5	5	5
4	10	10	10	7	4	4	4	4	5	5	5	5
5	10	10	9	10	4	3	4	3	5	5	5	5
6	10	10	10	10	4	3	4	3	5	5	5	5
7	10	10	9	9	4	4	4	4	5	5	5	5
8	10	10	9	10	4	4	4	4	5	5	5	5
9	10	10	10	9	4	4	4	4	5	5	5	5
10	10	10	10	10	4	3	4	3	5	5	5	5
11	10	10	7	8	3	3	3	3	5	5	5	5
12	10	10	10	10	4	4	4	4	5	5	5	5
Total. .	120	120	114	105	46	41	46	41	60	60	60	60

TABLE 2

SURVIVAL TIME IN DAYS: NELSON ASCITES MOUSE TUMOR

Experiment No.	Untreated controls										6-Mercaptopurine*									
	Mouse No.																			
	1	2	3	4	5	6	7	8	9	10	1	2	3	4	5	6	7	8	9	10
1	6	7	7	7	7	8	8	9	10	12	8	12	13	13	14	15	16	26	30	39
2	5	7	12	12	13	13	14	15	16	26	12	14	15	15	16	17	18	18	21	23
3	6	13	13	14	15	15	16	16	18	22	12	13	13	14	14	14	14	16	19	27
4	7	7	7	8	9	11	13	13	15	15	11	13	14	15	17	27	36	36	>42	>42
5	8	8	9	10	11	11	11	12	13	21	7	12	12	13	13	15	17	19	19	22
6	8	10	11	11	12	12	12	13	13	14	13	13	13	13	15	18	26	33	34	>42
7	7	8	8	9	10	10	14	14	14	21	7	11	12	14	14	14	17	21	24	37
8	9	11	12	12	12	12	13	14	15	18	12	13	13	14	16	16	23	23	35	>42
9	7	10	11	11	12	13	13	13	13	17	10	12	12	13	14	19	20	23	25	26
10	8	9	9	10	12	12	12	14	15	18	13	15	16	16	16	16	16	16	17	20
11	7	7	9	10	11	11	12	12	14	18	7	7	7	8	10	10	10	10	11	13
12	10	11	12	12	12	12	13	13	14	14	10	11	12	12	13	14	14	17	17	21

*Seven daily I.P. injections of 6-mercaptopurine (15 mg./kg.).

TABLE 3

TOTAL PACKED CELL VOLUME IN MILLILITERS ON THE SEVENTH DAY AFTER IMPLANTATION: NELSON ASCITES MOUSE TUMOR

Experiment No.	Untreated controls				6-Mercaptopurine*			
	Mouse No.							
	1	2	3	4	1	2	3	4
1	1.30	1.22	1.36	d†	0.45	0.07	0.48	d
2	2.11	1.50	d	d	0.33	1.22	0.88	0.47
3	1.86	1.02	1.32	2.78	1.34	0.02	1.16	0.00
4	1.71	1.23	1.76	1.71	1.28	0.00	0.54	0.65
5	1.49	1.91	1.51	d	0.00	0.10	0.59	1.43
6	1.94	2.63	1.85	d	0.85	1.56	0.74	0.54
7	2.04	1.95	2.55	1.99	0.19	0.24	0.68	0.34
8	1.02	1.88	1.33	1.68	0.78	0.35	0.77	0.00
9	1.84	1.93	1.68	1.30	0.31	1.31	0.69	0.61
10	2.19	1.47	2.19	d	0.85	0.32	0.91	0.56
11	2.05	0.00	2.49	d	0.36	0.02	0.14	d
12	1.98	1.58	1.45	1.86	0.89	1.12	0.00	0.74

* Seven daily I.P. injections of 6-mercaptopurine (15 mg./kg.).

† Died on or before the seventh day after implantation.

employed (an F ratio or the square root of F = "t" in terminology of the analysis of variance).

The first of these ratios is the local t test. This test gives a measure of local variation within the individual experiment expressed as the mean square (s^2) within a series of animals treated alike and in the same experiment. The results of the local t test for six quantitative indices are presented in TABLE 9.

TABLE 4

TOTAL BODY WEIGHT CHANGE (CARCASS AND TUMOR) FROM 0 TO 7 DAYS (IN GRAMS): NELSON ASCITES MOUSE TUMOR

Experiment No.	Mouse No.									
	1	2	3	4	5	6	7	8	9	10
1										
C*	+12.2	+11.8	+6.1	+11.9	+9.8	d‡	d	d	d	d
T†	+4.6	−1.2	+7.7	−1.2	+3.4	+0.6	+5.3	+3.8	−2.5	+5.9
2										
C	+4.5	+5.3	+5.7	+11.1	+4.0	+6.9	+10.6	+11.2	d	d
T	+0.3	+1.6	+2.7	+4.5	+7.3	+9.8	+9.6	+3.2	+3.3	+2.8
3										
C	+9.8	+10.4	+8.8	+7.4	+6.8	+12.1	+8.6	+5.5	+10.0	d
T	+7.0	+10.3	+6.3	+1.6	+10.5	+6.9	+12.5	+2.7	+6.2	+3.8
4										
C	+7.9	+6.3	+9.4	+10.1	+7.7	+10.9	+7.1	d	d	d
T	+3.1	−2.0	+2.5	+3.5	+7.0	+0.4	+5.1	−1.0	+4.7	+1.8
5										
C	+7.4	+12.8	+7.7	+11.2	+4.9	+8.7	+8.9	−2.8	+8.5	+7.9
T	+5.9	+0.3	+10.7	+9.9	+6.2	+4.8	+7.5	+3.5	−1.3	d
6										
C	+8.4	+10.7	+9.2	+10.2	+10.6	+11.5	+6.4	+8.2	+9.4	+7.7
T	+5.2	+1.8	+4.7	+2.9	+7.4	+7.5	+6.2	+7.8	+6.7	+6.7
7										
C	+13.0	+5.9	+12.4	+10.5	+13.9	+9.5	+7.3	+11.6	+4.5	d
T	+0.6	+9.2	+5.6	+7.7	−0.3	−2.6	+2.2	+0.7	+2.8	d
8										
C	+8.6	+10.6	+3.3	+8.3	+10.2	+8.2	+5.8	+8.5	+11.7	+3.5
T	+5.4	+0.7	+8.0	+5.1	+2.1	+3.2	+1.4	+2.2	+0.9	d
9										
C	+12.2	+6.0	+8.0	+11.1	+9.8	+7.5	+9.5	+5.9	+14.1	d
T	+5.7	+3.1	+4.9	+1.3	+1.0	+10.0	+5.5	+2.6	+2.5	+4.6
10										
C	+12.0	+11.9	+7.7	+12.1	+8.1	+8.1	+9.3	+9.6	+11.8	+8.0
T	+8.2	+5.5	+2.3	+3.4	+4.6	+2.5	+3.5	+6.6	+8.6	+4.4
11										
C	+9.3	+9.7	+9.8	+11.2	+6.3	+4.0	+11.3	+6.6	d	d
T	+8.0	+2.3	+9.3	+6.7	+1.3	−0.1	+4.6	d	d	d
12										
C	+9.5	+9.2	+9.8	+10.5	+7.6	+8.6	+6.7	+8.1	+7.7	+9.4
T	+6.9	−0.2	+7.0	+6.6	+6.1	+6.6	+4.7	+5.4	+5.5	+5.9

* Untreated controls.

† Treated with 6-mercaptopurine—7 daily I.P. injections (15 mg./kg.).

‡ Died on or before the seventh day after implantation.

The degrees of freedom can be found by subtracting one from the corresponding entry in TABLE 1.

It is evident that the change in carcass weight is an ineffectual index, since none of the 12 local t tests were significant at the 5 per cent level. In contrast, the total body weight change (carcass and ascites) shows 10 of the 12 t tests significant at the 5 per cent level. The remaining indices show 6 to 8 significant t tests out of 12.

The comparison of the proportions of significant local t tests often has been used to evaluate indices. Because the t values are dimensionless quantities, it is possible to use them directly to compare two indices with different dimen-

TABLE 5

CARCASS WEIGHT CHANGE FROM 0 TO 7 DAYS (IN GRAMS): NELSON ASCITES MOUSE TUMOR

Experiment No.	Untreated controls				6-Mercaptopurine*			
	Mouse No.							
	1	2	3	4	1	2	3	4
1	−5.1	−1.2	−4.1	d†	−1.6	−1.6	+0.9	d
2	+0.2	−2.8	d	d	+0.5	+0.1	+0.3	−0.8
3	−2.5	+6.7	+4.6	+1.8	+0.3	+1.0	−0.3	+2.2
4	+0.5	−1.0	+3.1	+1.1	−3.3	+1.6	+1.4	−0.2
5	+1.6	+0.9	+4.9	d	+4.3	−0.5	+2.2	+3.7
6	−1.8	−1.4	+1.8	d	+0.8	−0.1	+0.4	+1.1
7	+2.2	−0.6	+0.4	+1.8	−2.8	+1.2	+1.2	+0.4
8	+6.3	+0.8	+2.6	−2.2	−1.2	+1.4	−0.5	+1.0
9	−2.9	+2.3	−0.5	+3.4	−1.3	+1.6	+2.1	+4.5
10	+1.2	−1.9	−0.5	d	+0.1	−2.4	−0.6	−1.3
11	−0.6	+3.0	+2.4	d	−0.3	−1.7	−1.3	d
12	−1.0	−1.9	+3.7	−1.3	−0.3	−1.1	−0.4	+1.2

* Seven daily I.P. injections of 6-mercaptopurine (15 mg./kg.).

† Died on or before the seventh day after implantation.

TABLE 6

TUMOR DIAMETER ON THE SEVENTH DAY AFTER IMPLANTATION (IN CENTIMETERS): NELSON ASCITES MOUSE TUMOR, SOLID FORM

Experiment No.	Untreated controls					6-Mercaptopurine*				
	Mouse No.									
	1	2	3	4	5	1	2	3	4	5
1	1.45	1.40	1.60	1.50	1.25	1.20	0.75	1.50	1.07	0.95
2	1.17	1.32	1.15	1.15	0.85	0.85	0.77	0.95	1.20	1.00
3	1.75	1.70	1.65	1.40	1.40	1.40	1.48	1.60	1.00	1.18
4	1.55	1.45	1.20	1.15	1.45	1.00	1.00	1.05	0.87	0.80
5	1.30	1.75	1.40	1.35	1.30	1.23	1.00	1.25	1.10	1.30
6	1.80	1.65	1.70	1.75	1.63	1.17	1.05	1.17	0.87	1.10
7	1.35	1.40	1.50	1.40	1.65	1.05	0.80	1.30	1.00	1.20
8	1.50	1.77	1.75	1.20	1.82	1.30	1.15	1.00	1.60	1.52
9	1.50	1.55	1.25	1.75	1.60	1.38	1.17	1.07	1.20	1.07
10	1.65	1.42	1.25	1.80	1.42	0.98	0.85	1.00	1.15	1.32
11	1.82	1.75	1.65	1.45	1.87	1.68	0.80	1.60	0.85	0.92
12	1.65	1.60	1.55	1.65	1.90	1.40	1.30	1.42	1.20	1.02

* Seven daily I.P. injections of 6-mercaptopurine (15 mg./kg.).

sions. This approach is sanctioned by the textbooks and would meet the approval of many statisticians.

There is some question, however, as to whether this is a good criterion to use. In practice, the local t test is sometimes erratic and leads to spurious significances more often than the 5 per cent level would indicate. The local variation may grossly underestimate the experimental error, especially when there are fewer than 10 degrees of freedom available for estimating the error within

TABLE 7

TOTAL BODY WEIGHT CHANGE (CARCASS AND TUMOR) FROM 0 TO 7 DAYS (IN GRAMS): NELSON ASCITES MOUSE TUMOR, SOLID FORM

Experiment No.	Untreated controls					6-Mercaptopurine*				
	Mouse No.									
	1	2	3	4	5	1	2	3	4	5
1	+5.0	+4.8	+6.9	+6.4	+3.8	−0.5	−5.7	+4.0	−1.1	−4.9
2	+4.3	+10.2	+1.8	+8.3	−1.0	−2.9	−5.8	+2.3	+3.0	+2.7
3	+8.6	+7.2	+8.3	+10.7	+6.8	+5.5	+3.6	+5.9	+2.1	+5.0
4	+7.6	+8.0	+8.1	+6.6	+7.0	+2.0	+2.6	+1.5	−1.2	+3.8
5	+4.5	+8.3	+5.4	+4.0	+4.1	+1.8	+3.4	+4.6	+3.9	+2.7
6	+6.6	+6.5	+7.0	+7.2	+7.5	+6.2	+2.5	+6.1	+1.2	+2.1
7	+7.2	+6.7	+8.0	+7.5	+8.8	+2.5	+3.5	+4.8	−1.5	+5.9
8	+3.2	+8.5	+4.7	+6.2	+6.2	+6.1	+2.6	+3.6	+4.6	+5.6
9	+8.3	+7.0	+4.4	+6.1	+5.5	+5.3	+4.1	+2.6	+2.7	+2.0
10	+10.8	+9.6	+8.0	+9.4	+9.1	+4.0	+1.4	+2.1	+2.8	+4.8
11	+6.0	+7.4	+8.6	+5.1	+8.1	+6.5	+5.9	+6.3	+3.9	+2.6
12	+6.6	+5.2	+4.9	+6.4	+7.8	+3.8	+2.9	+4.3	+2.8	+3.0

* Seven daily I.P. injections of 6-mercaptopurine (15 mg./kg.).

TABLE 8

MEANS OF SERIES: SIX INDICES

Experiment No.	Survival time (1)		Body wt. change—car. and tumor (2)		TPCV (3)		Carcass wt. change (4)		Tumor diameter (5)		Total body wt. change—car. and solid tumor (6)	
	T	C	T	C	T	C	T	C	T	C	T	C
1	18.6	8.1	2.64	10.30	0.33	1.29	−0.77	−3.47	1.09	1.44	−1.64	5.38
2	16.9	13.3	4.50	7.40	0.72	1.80	0.02	−1.30	0.95	1.12	−0.14	4.72
3	15.6	14.8	6.78	8.82	0.84	1.74	0.80	2.65	1.33	1.58	4.42	8.32
4	23.5	10.5	2.51	8.48	0.82	1.60	−0.12	0.92	0.94	1.36	1.74	7.46
5	14.9	11.4	5.28	7.52	0.53	1.64	2.42	2.46	1.18	1.42	3.28	5.26
6	22.0	11.6	5.69	9.23	0.92	2.14	0.55	−0.46	1.07	1.70	3.62	6.96
7	17.1	11.5	2.88	9.84	0.36	2.13	0	0.95	1.07	1.46	3.04	7.64
8	20.7	12.8	3.22	7.87	0.48	1.48	0.18	1.88	1.31	1.60	4.50	5.76
9	17.4	12.0	4.10	9.34	0.73	1.69	1.72	0.58	1.18	1.53	3.34	6.26
10	16.1	11.9	4.96	9.86	0.66	1.95	−1.05	−0.40	1.06	1.50	3.02	9.38
11	9.3	11.1	4.59	8.52	0.17	1.51	−1.10	1.60	1.17	1.70	5.04	7.04
12	14.1	12.3	5.45	8.71	0.69	1.72	−0.15	−0.12	1.26	1.67	3.36	6.18
Over-all mean..	17.3	11.7	4.38	8.82	0.60	1.72	0.20	0.44	1.13	1.50	2.79	6.69

the experiment. Such a situation arises when, for example, as few as four animals per series are used for measuring an index.

For this reason we have considered also a sensitivity/stability ratio over time (TABLE 10). This ratio was calculated directly from 12 differences between the mean of the treated series and the mean of the control series in an experiment. The variation over time (in the denominator of the ratio) serves as

TABLE 9

LOCAL T TESTS: SIX INDICES

Experiment number	Survival time (1)	Body wt. change—car. and tumor (2)	TPCV (3)	Carcass wt. change (4)	Tumor diameter (5)	Total body wt. change—car. and solid tumor (6)
1	3.32*	4.34*	6.88*	1.70	0.88	3.86*
2	1.74	6.04*	3.03†	2.55	1.60	1.80
3	0.41	1.52	1.70	0.91	0.19	4.12*
4	3.55*	4.93*	2.69†	0.74	4.77*	6.52*
5	1.90	1.18	2.77†	0.02	2.38†	2.13
6	2.97*	4.32*	1.16	1.36	10.00*	3.15†
7	1.85	4.12*	9.94*	0.82	3.82*	3.48*
8	2.35†	3.78*	3.73*	0.92	1.84	1.16
9	2.63†	4.19*	3.47†	0.62	3.54*	1.35
10	3.72*	5.27*	0.50	0.06	3.64*	4.25*
11	1.48	2.46†	1.72	2.26	2.61†	2.00
12	1.58	4.23†	3.77*	0.02	1.66	4.77*
Average t	2.29	3.86	3.45	0.99	3.08	3.22
Total significant at 5 per cent level	6	10	8	0	7	7
Per cent significant at 5 per cent level	50	83	61	0	58	58
Ranking by per cent significant	5	1	2	6	3.5	3.5

* T values significant at the 1 per cent level.
† T values significant at the 5 per cent level.

TABLE 10

TIMEWISE T TESTS: SIX INDICES (TREATMENT × EXPERIMENT INTERACTION)

Factor	Survival time (1)	Body wt. change—car. and tumor (2)	TPCV (3)	Carcass wt. change (4)	Tumor diameter (5)	Total body wt. change—car. and solid tumor (6)
T test	4.15	8.57	14.93	1.02	13.77	7.15
Significance	*	*	*	†	*	*
Ranking	5	3	1	6	2	4

* T values significant at the 1 per cent level.
† Not significant.

the measure of the stability of an index. The relative performance of the six indices shows a somewhat different ranking. The carcass weight change is still the poorest index, but the TPCV and tumor diameter now appear to be relatively better indices than the total body weight change.

While the two sensitivity/stability ratios represent a good way to consolidate the experimental data into a single number, they do not provide a graphic picture of the behavior of the indices. In order to present the results in a form sufficiently graphic to be intercompared, a technique called the Ridit Analysis* has been employed (see APPENDIX 2).[4]

* Ridit = Relative to Identified Distribution.

Thus far the comparison between the indices has been made in such terms of the original scale as days, grams, and centimeters. It is possible to transform all the original indices so as to be able to plot them on the same comparable scale, using as a measure of drug effects the probability that a treated animal will be better off than a control animal.

In brief, the procedure of a Ridit Analysis is as follows:

(1) All the control observations in the twelve experiments are pooled to give the "identified distribution" of the observations for a particular index.

(2) By cumulating the frequencies in the "identified distribution," we calculate: ridit for Y_i = (proportion of observations less than Y_i) + ½ (proportion of observations equal to Y_i). Irrespective of the dimension or nature of the original scale (Y), the ridits will be dimensionless quantities on a 0 to 1 scale.

(3) The process described in point 2 is called a transformation. This ridit transformation is now applied to the observation in the positive control as well as those in the negative control series, and average ridits are calculated. Then, for example, an average ridit of 0.75 for survival time in the treated series would mean that a treated animal has an estimated 3 chances of 4 of surviving longer than an untreated animal. An average ridit of 0.75 with respect to tumor diameter would mean that a treated animal would have an estimated 3 chances of 4 of having a smaller tumor than an untreated animal. Hence the average ridit has a similar interpretation for all of the indices, so that direct comparisons of ridits are meaningful.

FIGURES 1 through 6 show the results for the Ridit Analysis of six tumor inhibition indices in the present study. If the index is stable over time, its

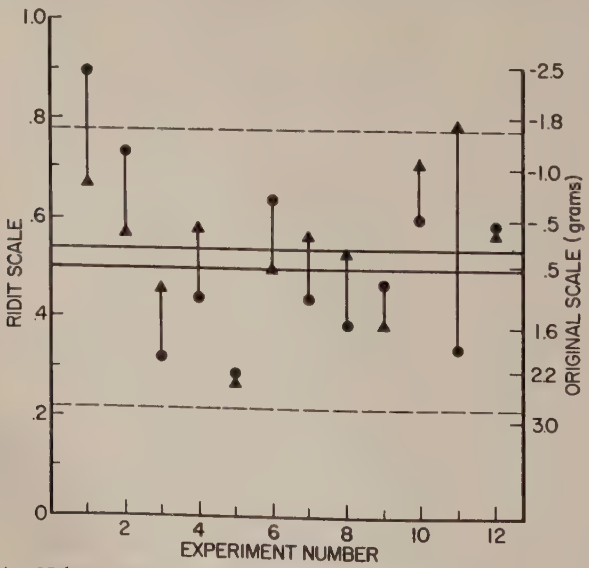

FIGURE 1. Nelson ascites tumor: carcass weight change (0 to 7 days) in grams.

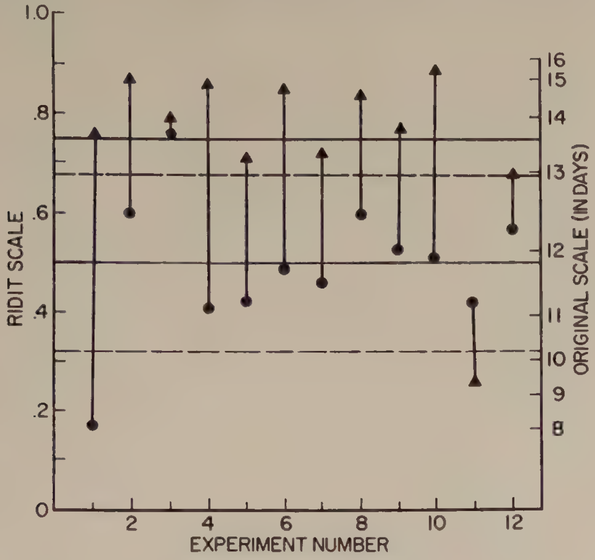

FIGURE 2. Nelson ascites tumor: survival time in days.

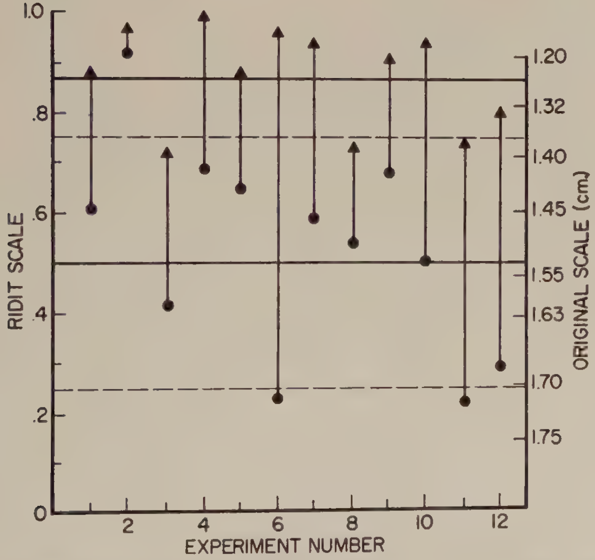

FIGURE 3. Nelson ascites tumor—solid form: diameter (7 days after implantation) in centimeters.

variation over time should be no larger than local variation, and nearly all the mean values of control indices of the individual experiment indicated by circles in the charts should fall between the upper and lower control limits. These approximate control limits (the two dotted horizontal lines on the ridit charts)

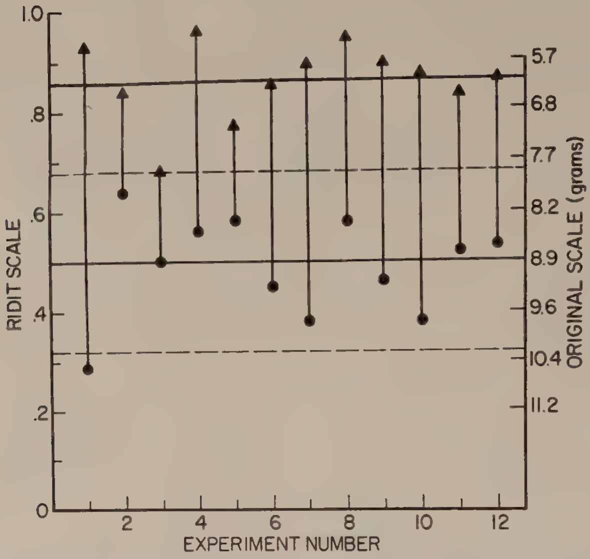

FIGURE 4. Nelson ascites tumor: total body weight change (carcass and tumor—0 to 7 days) in grams.

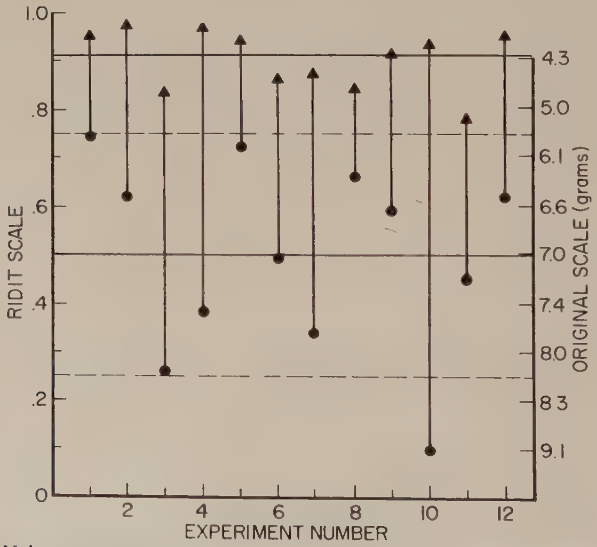

FIGURE 5. Nelson ascites tumor—solid form: total body weight change (carcass and solid tumor—0 to 7 days) in grams.

extend two standard errors from the central value of one half. The number of experiments inside the control limits is a rough measure of the stability of an index.

The abscissa of the Ridit charts really represent time, since experiments 1 through 12 are plotted in their temporal sequence.

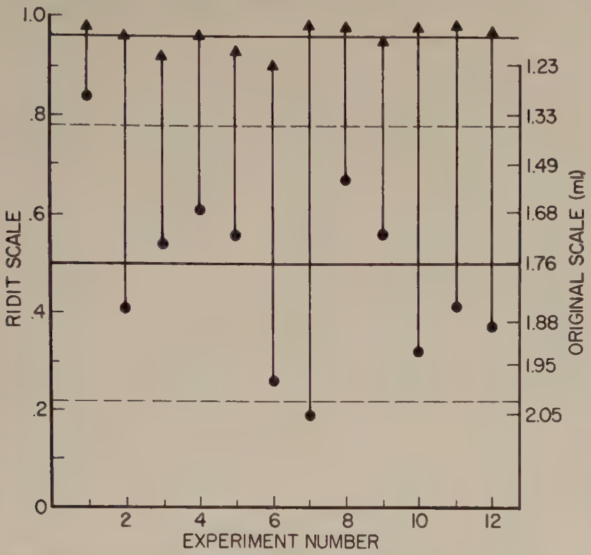

FIGURE 6. Nelson ascites tumor: total packed cell volume (seventh day) in milliliters.

Of the untreated control values for carcass weight change (FIGURE 1), all but one lie within the control limits band, indicating the stability of this index. However, all the values for 6-MP series (indicated by triangles) fall below the upper control limit of the untreated series, which shows the very poor sensitivity of this index.

The survival time (FIGURE 2) and tumor diameter (FIGURE 3) values of the control series show some instability, since two of the control observations of the former index and three of the latter fall outside the control limit band. These two indices also are moderately sensitive—only two 6-MP values for the survival time, and three for the tumor diameter fall inside the central band.

The total body weight changes for the fluid and solid tumors (FIGURES 4 and 5) show good stability and sensitivity, and a good separation of the 6-MP from the control values.

In the case of TPCV (FIGURE 6), two control values lie outside the central band, so that this index is not exceptionally stable. However, all the 6-MP values lie well above the central band and, in fact, all of the average ridits are greater than 0.9. The average of the 6-MP series is above the ridit of 0.95. This means that there is a 19-to-1 chance that a TPCV of the 6-MP series will be less than the corresponding TPCV from the control series. This marked separation is achieved on groups of only 4 mice, compared to 5 for solid tumor and 10 for survival time of the ascites tumor-bearing animals.

A summary of the rankings obtained by means of the local t test, of the sensitivity/stability ratio over time, and of the Ridit Analysis is given in TABLE 11. It will be seen that, by and large, these three various approaches have led to a reasonably concordant conclusion that the TPCV tends to lead the list, while the carcass weight change falls at the bottom of the list. These

TABLE 11

SUMMARY OF RELATIVE RANK: SIX INDICES

Measure	Survival time (1)	Body wt. change—car. and tumor (2)	TPCV (3)	Carcass wt. change (4)	Tumor diameter (5)	Total body wt. change—car. and solid tumor (6)
Local t test....	5	1	2	6	4	3
Timewise t test.	5	3	1	6	2	4
Average ridit (6-MP)	5	4	1	6	3	2
Over-all.......	5	3	1	6	3	3

three standards of evaluation by no means exhaust the list of possible criteria for comparing screening indices, and such criteria as variances, coefficient of variation, and control charts in the original scale also could be used. However, we feel that the three standards utilized here gave results sufficiently concordant to provide a good all-around picture of the performance of indices.

Summary

With the aim of establishing their potential value in a cancer chemotherapy screening program, the performance of six different indices of tumor growth was compared.

The Nelson ascites mouse tumor growing in ICR albino mice was tested in both the ascites and solid forms.

The six indices included: survival time in the ascites form, total body weight change in the ascites and solid forms, carcass weight change in the ascites form, diameters of solid tumors, and the total packed cell volume of the ascites form.

Twelve individual experiments were performed over a period of seventy days. The above indices were obtained in each experiment for "untreated" controls and for the groups of mice receiving intraperitoneal injections of 6-mercaptopurine.

The six indices were compared with respect to three different measures of performance: (1) the standard t tests for control versus 6-mercaptopurine calculated for each index in each experiment (= local t test); (2) the sensitivity/stability ratio for each index over time, using the "treatment × experiment" interaction as the experiment error; and (3) a transformation of each index to a Ridit Scale, where direct graphic comparisons may be made.

These three approaches have led to a reasonably concordant conclusion that the total packed cell volume heads the list of indices, the carcass weight change tends to fall to the bottom of the list, and the remaining four indices occupy the intermediate ranking positions.

APPENDIX 1

The data on 3-animal series (20-day survival) were not included in the analysis in the results section because, unlike the other indices, they were not quantitative. The purpose of carrying the 3-animal series was to test a simple sequential screening technique suggested by Bross.

The details of this scheme will not be given, since the method did not work. However, the basic idea was to run 3 animals on a test agent and, if any survived 20 days, to run another 3 animals (and so on until a maximum of 9 animals were tested on the drug). The scheme was based on data from previous experiments in which it was found that relatively few untreated control animals survived 20 days, whereas a sizable proportion of 6-MP animals did survive for 20 days.

The main reason for the failure of the scheme in the data reported here was that the survival time for 6-MP animals was much poorer in this experiment than in the previous ones. Consequently, the scheme did not discriminate between the untreated and treated controls. We do not know why the survival time became a less sensitive index. Indeed, had it not been for the dual controls carried in these experiments, we would not have even been aware of this loss of sensitivity.

It is worth noting that, although the survival time for the treated controls was lower than in previous experiments, there was no corresponding drop in the survival times for the untreated controls. Also, there was no warning from statistical theory that the scheme would not work; it worked well on paper and in a retrospective trial on the earlier data.

One important conclusion can be drawn from this negative result. The acid test for a statistical method is its performance in actual screening operations—theory is no substitute for an empirical test. Moreover, there must be a continuing check on the method, since the sensitivity of the screen may fade. This is one reason why dual controls are likely to be useful in screening operations.

APPENDIX 2

The details of Ridit Analysis are explained in a paper by Bross.[4] As an illustration of the application of this technique to screening data, the computational process for the ridits for survival time is given in TABLE 12.

After the ridits are calculated, the observations in the treated series are converted from the original scale to the ridit scale. Averages and other statistics may then be calculated in the usual way for each series.

Ridits merit consideration as an analytic tool in mass screening because they possess a

TABLE 12
CALCULATION OF RIDITS (COMPUTING FORM)

Survival time in days	1	2	3	4	5
5	1	0.5	0	0.5	0.0042
6	2	1.0	1	2.0	0.0166
7	12	6.0	3	9.0	0.0750
8	9	4.5	15	19.5	0.1625
9	8	4.0	24	28.0	0.2333
10	9	4.5	32	36.5	0.3042
11	12	6.0	41	47.0	0.3916
12	21	10.5	53	63.5	0.5292
13	16	8.0	74	82.0	0.6833
14	11	5.5	90	95.5	0.7958
15	7	3.5	101	104.5	0.8708
16	3	1.5	108	109.5	0.9125
17	1	0.5	111	111.5	0.9291
18	4	2.0	112	114.0	0.9500
21	2	1.0	116	117.0	0.9750
22	1	0.5	118	118.5	0.9875
26	1	0.5	119	119.5	0.9958
Total.............	120		120		

Instructions:
Column 1: frequency distribution in the identified distribution (reference class).
Column 2: one-half of the corresponding entry in Column 1.
Column 3: cumulate of Column 1 (displaced one category downward).
Column 4: Column 2 and Column 3.
Column 5: entries in Column 4 divided by grand total (120). The numbers are the ridits.

number of potentially valuable properties. Whether they will be useful in a given screen is something that can be determined only by trial. Some additional computation is required at the beginning (and some useful auxiliary information is gleaned in the process) but, in the long run, there is less than the usual amount of calculation. Like other ranking methods, Ridit Analysis leads to "distribution-free" procedures (which means that some of the usual assumptions can be avoided). The following are some of the properties of ridits:

(1) They are simple in the technical sense that such things as control charts and significance tests are comparatively easy to construct and use.

(2) The average ridit in the treated series, being relative to a pooled control series, brings in relevant past experience that is currently ignored in statistical procedures.

(3) The average ridit in the treated series can be compared to the average ridit for the untreated control for the same experiment by using the formula: treated − control + ½. This is an estimate of the probability that an animal in the treated series will be "better off" than an animal from the population represented by the local control.

(4) If a dual control is carried (that is, a "standard" agent is also included) then a second set of ridits with the standard data for the identified distribution can be calculated. The average standard ridit in a test series estimates the probability that an animal treated with the test agent will be "better off" than an animal given the standard agent. This may be a more meaningful way to describe the performance of an effective agent (that is, relative to a known effective agent rather than to a dummy agent). Control charts and significance tests also can be based on the standard ridit.

(5) Ridits have some interesting stability properties that may improve the comparability of reported results. For example, suppose two investigators start from the same data, but one prefers to work in terms of a "linear" dimension (x) while the other prefers a "volume" measure (x^3). If both convert to ridits, they will obtain exactly the same numerical answers.

(6) Ridits can be used even when the response variable is not numerical (for example, if a subjective scale is used).

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TOTAL PACKED CELL VOLUME AS A MEASURE OF GROWTH RETARDATION IN MOUSE ASCITES TUMORS*

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Tarnowski's presentation of a comparative study of a number of indices of acites tumor growth with reference to their applicability in routine screening represents a recognition of the desirability of quantification of ascites tumor-screening procedures. To allow more ready interpretation of results and more valid comparison with results from existing screening systems, such a quantification might optimally employ criteria with characteristics comparable to those of the more classic screens.

In the past, the quantitative criteria most used in screening in the tumor-bearing animal have been (1) retardation or reduction of primary tumor growth and (2) extension of survival time of the tumor-bearing host. The first of these is the more direct measure of inhibition effects against the cancer cell per se, but requires a reliable measure of the amount of primary tumor growth. In the case of ascites tumors, this measurement of the total amount of tumor cell material in the ascitic fluid of the host has been made most frequently in terms of total cell number, but also may be made in terms of total cell volume, a property that may be considered comparable to that of volume or weight of solid tumors. Quantitative determination of this cellular fraction of ascites tumors as total packed cell volume (TPCV) has proved to be both rapid and reproducible under routine screening conditions.

Determination and Characterization of the TPCV Parameter

TPCV can be calculated simply as the product of the packed cell volume percentage, or ascitocrit (determined in capillary tubes in a microhematocrit centrifuge),[1] and the total amount of ascitic fluid (determined as the difference between the weight of the animal plus tumor and the body weight of the sacrificed, drained animal). FIGURE 1 shows a comparison of TPCV with other parameters of tumor growth for one line of the Ehrlich ascites tumor. In the lower plot of volume measurements in FIGURE 1, the total ascitic volume is seen to increase most rapidly during the period in which the ascitocrit drops sharply. The product of these two measurements gives the resultant TPCV curve, which follows a more gradual rise to a final leveling-off and decrease. The cell count measurements in the upper plot of FIGURE 1 indicate an almost linear increase in total cell number during the intermediate stage of tumor growth, with a concomitant increase in percentage of nontumor cells, which rises sharply during the latter stage of tumor growth. Correcting the total cell count curve for the nontumor cells results in the calculated curve for total number of tumor cells, which parallels closely the more easily determined TPCV curve for total volume of ascitic cellular material.

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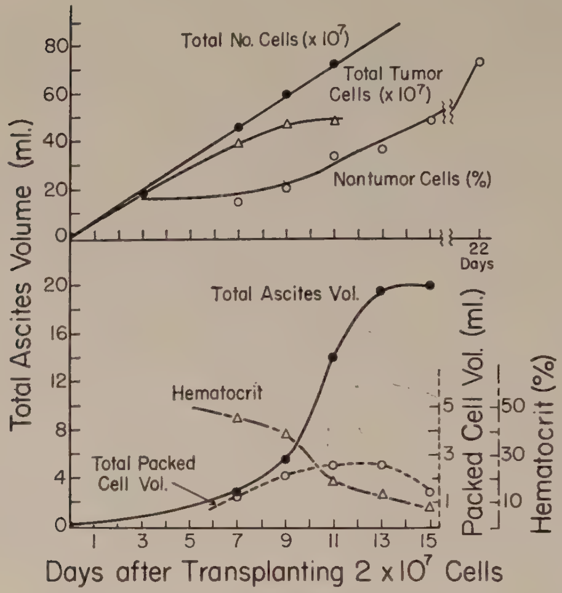

FIGURE 1. Comparison of growth parameters for one line of the Ehrlich ascites tumor The lower plot represents volume measurements; the upper plot, cell count measurements.

The quantitative application of any parameter of ascites tumor growth such as TPCV to routine screening requires knowledge of the specific growth characteristics for each individual tumor-host system used. FIGURE 2 shows TPCV growth curves for five ascites tumors under standardized conditions of inoculation. It is apparent here that, for valid determination of retardation effects on TPCV, assessment of the effectiveness of treatment must be made before the TPCV curve passes through a maximum and becomes negatively accelerating. For the faster-growing lymphomas, then, treatment may be continued during the so-called log phase of tumor growth up to 6 days following tumor inoculation, with assessment of treatment—or termination of the screening procedure—on day 7. For the slower-growing Ehrlich and hepatoma tumors, treatment can be continued 1 day longer, with termination of the screening procedure on day 8.

To separate true carcinostatic effects of drug treatment from tumor retardation due to drug toxicity, it is necessary to know also the effects of body weight loss per se on the TPCV parameter for each tumor-host system used. By controlled feeding experiments it is possible to acquire the sort of information summarized in FIGURE 3. Here, the extent of tumor growth (as percentage of mean TPCV of ad libitum-fed controls) has been plotted against grams of body weight loss for individual tumor-bearing animals, and the resultant lines of best fit for such data for 6 ascites tumors are shown. The intercept of each line at 100 per cent control growth represents the weight-loss limit which, of itself, does not affect the TPCV parameter for that tumor. This allowable weight-loss limit can be seen to vary from 2.5 gm. for the most sensitive Ehrlich

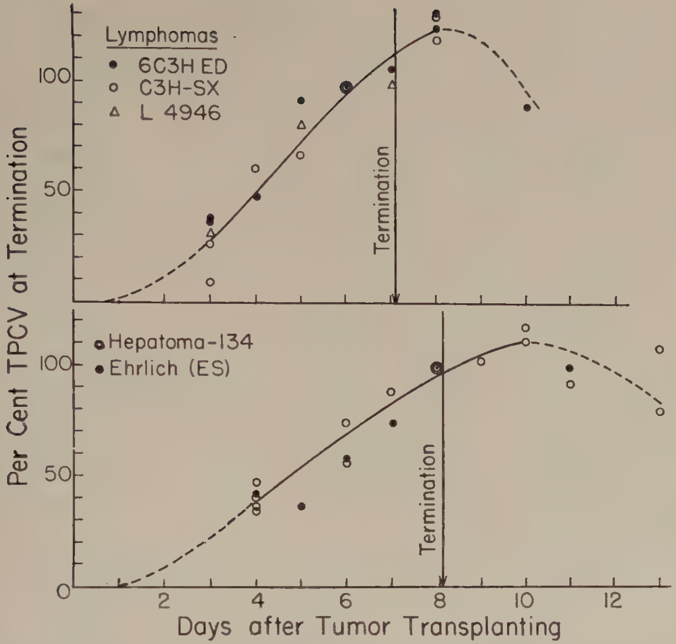

FIGURE 2. TPCV growth curves for 5 ascites tumors under standardized screening conditions. The data for each tumor are taken from several experiments; each point represents the average of 5 replicate animals. Tumor growth curves are equated on the basis of growth rates, by expressing TPCV values within each experiment as the percentage of the TPCV value on the termination day in a routine screening procedure.

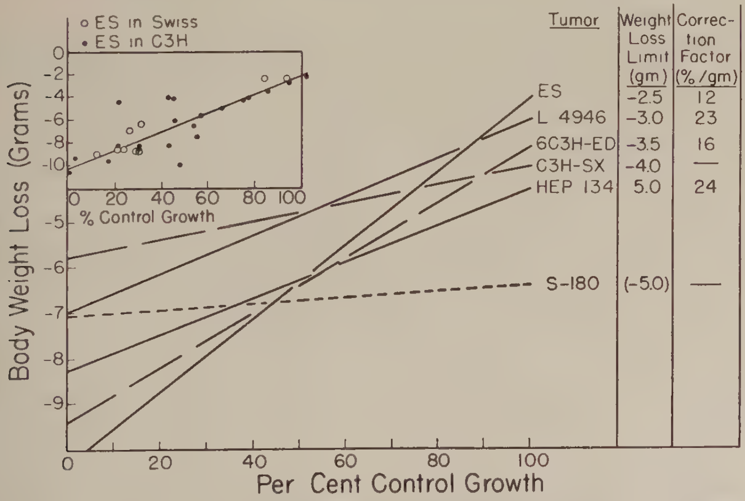

FIGURE 3. Effect of body weight loss on the TPCV parameter for 6 ascites tumors. TPCV reduction was effected by food deprivation, which paralleled the schedule of drug treatment under screening conditions. The extent of tumor growth for individual animals, expressed as the percentage of ad libitum-fed controls, was plotted against grams of body weight loss for individual animals; the line of best fit was determined by the method of least squares. Representative data for the Ehrlich (ES) tumor are shown in the small inset plot. Tumor abbreviations refer to the ascites tumors listed in TABLE 1.

to more than 5.0 gm. for the nonaffected Sarcoma 180 ascites. The slope of each line (which may be used as an approximate correction factor for weight losses beyond the indicated limit) varies from 12 per cent per gram for the Ehrlich to a practically indeterminate figure for the Sarcoma 180 and the C3H-SX lymphoma. It is of interest to note that both the Sarcoma 180 and the Hepatoma 134 ascites tumors, which tolerate the greatest induced body weight loss without reduction in final TPCV, possess the property of causing progressive body weight loss in the host during the normal course of untreated tumor growth.

In actual practice we have characterized a number of ascites tumors as indicated, and have used them appropriately in a screening procedure[2] patterned after the classic screens with solid tumors. This procedure includes: (1) transplantation of a standardized inoculum (4 to 15 million cells) for each tumor (that is, the equivalent of 0.05 ml. of a characteristic 7-day-old tumor); (2) daily treatment by intraperitoneal (I.P.) injection during the period of maximal tumor growth; (3) measurement of individual TPCV values in treated and control groups one day after the last injection; and (4) assessment of effectiveness of treatment by comparison of mean TPCV values between treated and control groups. In more rigorous studies employing a sequential analysis screening design, mean log TPCV values of treated and control groups are compared.

Comparative Studies with the TPCV Criterion in an Ascites Tumor Screen

Ascitic versus solid tumor forms. In preliminary tests with both solid and ascites tumors it was found that the TPCV measure of the ascites tumors was less variable than the comparable parameter of tumor weights for the solid tumors tested. Table 1 shows a comparison of these measures of tumor size for 6 ascites tumors and 4 solid tumors under comparable screening conditions. A simple measure of the variability within control groups is shown as "coefficients of variation" (standard deviation ÷ mean), which vary from 22 to 36 per cent for TPCV values and from 38 to 56 per cent for solid tumor weights.

Lower variation allows the use of a smaller group size in screening experiments and/or the acceptance of a smaller reduction in average tumor size for the same level of statistical significance. Thus, for comparative purposes, the last column in table 1 shows the approximate group size for each tumor required to detect 50 per cent inhibition of tumor growth in a single test calculated under certain simplifying assumptions and within preset statistical limits of error. These group sizes do not represent the most efficient distribution of animals in a screening experiment under the assumptions made, but they illustrate the relative economy that can be effected with the less variable tumor systems.

Table 2 summarizes the chemotherapeutic responses of solid and ascitic forms of the same neoplasms to treatment with several drugs under routine screening conditions. The assessments of + and − responses have been equated between solid and ascites tumors on the basis of comparable levels of significance. Here again the ascitic forms proved less variable: a 25 per cent

TABLE 1

VARIATION OF CONTROL TUMORS UNDER ROUTINE SCREENING CONDITIONS

Ascites tumors	Average TPCV* (ml.)	±Standard deviation	Per cent coeff. of variation†	N for 50 per cent reduction‡
Ehrlich (ES)	1.53	0.47	33	7
Sarcoma 180	0.82	0.25	30	7
Hepatoma 134	1.75	0.38	22	4
Leukemia 4946	0.36	0.12	33	8
Lymphosarcoma 6C3HED	0.25	0.09	36	9
Lymphoma C3H-SX	0.28	0.08	29	6
Solid tumors	**Average weight (gm.)**			
Carcinoma C3H-BA	1.86	1.02	55	21
Carcinoma C3H-S	2.69	1.01	38	10
Carcinoma A-T3	1.32	0.59	45	14
Sarcoma 180	0.70	0.395	56	22

* Average of 5 to 15 separate control groups per tumor.

† Coefficient of variation = standard deviation ÷ mean.

‡ Calculated minimum group size to detect 50 per cent reduction in tumor size, assuming equal group size and equal variation for treated and control groups, and allowing 10 per cent error for false positives and 5 per cent error for false negatives. $N = \frac{(2.925)^2 \times 2 \times s^2}{(0.5\ \bar{X})^2}$ where N = minimum group size, s = standard deviation among individuals, and $\bar{X}$ = mean tumor size of control groups. This calculation does not contain correction for small sample statistics.

reduction in TPCV for the ascites tumors in groups of 10 was significant at the 5 per cent level of confidence, while a 40 per cent reduction in tumor weights was required to approach the same statistical significance with the encapsulated solid tumors in groups of 15.

On this basis of equating responses, the ascitic forms proved to be equally sensitive or more sensitive to treatment with the drugs tested, compared to the solid forms of the same neoplasms. These findings agree with those of Sugiura in his work with the two forms of the Ehrlich and Sarcoma 180 tumors, and with his general conclusion that, in practically all cases, the ascitic form is the more sensitive to positive carcinostatic agents.[3]

TPCV reduction versus survival extension. Although Patt has already pointed out that survival is an indirect measure of ascites tumor growth,[4] survival extension is the quantitative criterion most used in past ascites tumor screens. If one again makes certain assumptions (that is, that tumor growth retardation occurs only during the course of treatment and that the normal growth rate is resumed at the termination of treatment), then from the TPCV growth curves of FIGURE 2 an arbitrary relationship can be set up between survival extension and TPCV reduction. For the lymphomas, for example, a tumor that had achieved a TPCV value of 25 per cent of controls at the termination of treatment should have been retarded 4 days in its growth and should demonstrate a 4-day extension in survival. Similarly, one that had attained

TABLE 2

EFFECTS OF SELECTED CARCINOSTATIC AGENTS ON FOUR TRANSPLANTABLE MOUSE NEOPLASMS IN THE ASCITIC AND SOLID FORMS

Agent	Daily I.P. inj. (mg./kg.)	Neoplasms							
		Ehrlich		C3H-SX		6C3HED		S-180	
		Solid	Ascites	Solid	Ascites	Solid	Ascites	Solid	Ascites
Methyl bis(β-chloroethyl)amine	0.45	±	+	−	+	±	+	−	+
1,2-Bis(β-chloroethylthio)ethane	4.50	−	+	−	+	−	+	−	
N,*N*′,*N*″-Triethylenethiophosphoramide	2.25	±	+	±	±	±	+	±	+
1,4-Dimethanesulfonyloxybutane	11.20	−	−		−	−	±	−	±
Urethan	450.00	±	+	±	±	±	±	±	±
8-Azaguanine	45.00	−	+	−	−	±	+	−	±
6-Mercaptopurine	56.00	±	±	−	−	−	±	+	+
Thioguanine	3.40	−	+		+	−	+	+	+
Aminopterin	0.27	−	+	−	+	±	±	−	+
Azaserine	3.40	±	+	±	+	+	+	+	+
Deoxypyridoxine	112.00			−	+	−	−	−	±
Hydrocortisone	25.00			±	+	−	±	±	+

Effect	Per cent control growth*		Approx. level of significance† (P value)
	Ascites	Solid	
−	75–100	60–100	>0.10
±	50–75	30–60	0.01 – 0.05
+	0–50	0–30	0.001

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* Measured as TPCV for the ascites tumors and as tumor weight for the solid tumors.

† Approximate P value for the difference between treated and control groups for the indicated reduction in tumor growth.

a value of 50 per cent of controls at termination of treatment should evidence an extension of survival of about 2.5 days. This calculated relationship is shown in FIGURE 4 by the heavy line joining the open circles. The points plotted indicate the actual response of the 6C3HED lymphoma ascites to treatment by 44 selected agents. It is apparent that few agents fall near the theoretical curve, and that the majority of agents producing a significant effect by the TPCV criterion produce little or no effect on survival extension. In general, a similar picture is presented by the C3H-SX lymphoma, the L 4946 leukemia, and Sarcoma 180 ascites tumors.

These results are not surprising, and they serve principally to accentuate the error of our basic assumption that survival is a direct function of primary ascites tumor growth. In the case of the lymphomas, host death appears to be mediated by way of systemic invasion of the organism and, in the case of the S-180 line studied, death is due at least partially to a rapidly induced reduction of food intake with concurrent progressive loss of body weight (E. N. Sassenrath and F. M. Costa, unpublished observations). Survival measurements also are subject to the error of possible indeterminable drug toxicity effects

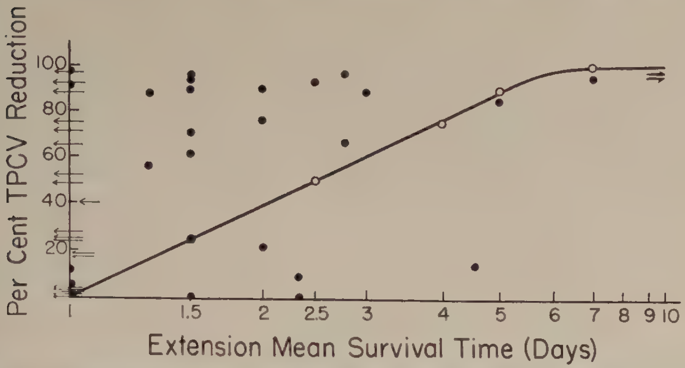

FIGURE 4. Comparison of the TPCV and survival criteria for the action of 44 selected agents on the 6C3HED ascites tumor. The solid line (joining the open circles) represents a theoretical relationship between TPCV reduction and survival extension, which is calculated from the upper growth curve of FIGURE 2 and based on the assumptions stated in the text. Each point represents the response of the tumor to treatment by a single drug under parallel screening conditions for both criteria. Arrows indicate the points that lie outside the survival extension scale.

that, at maximum tolerated levels, can operate to counteract positive treatment effects in screening.

In general, then, it can be said that, for the tumors and agents studied here, TPCV appears to be a more sensitive criterion than survival extension in an ascites tumor screen. It should be noted, however, that, as shown in FIGURE 4, a few agents produce a pronounced extension of survival with little or no effect on the TPCV criterion as evaluated immediately after cessation of treatment. To some extent, the small percentage of agents that show this is probably the result of the preponderant use of growth-retardation criteria in the screening procedures that have detected our present list of so-called carcinostatic drugs. Moreover, the occurrence of even a few such response patterns indicates the necessity of viewing the TPCV and survival criteria as independent measures that may be most profitably applied concurrently.

Specificity of TPCV response of six ascites tumors to chemotherapy. The existence of characteristic "profiles of sensitivity" to chemotherapy among individual ascites tumors, and even among different lines of the same parent tumor, has been amply demonstrated.[3, 5] In a current survey with about 40 agents, my associates and I have extended this observation in parallel tests with the TPCV criterion on 6 ascites tumors. Of the tumors tested—Ehrlich (ES), Hepatoma 134, Sarcoma 180, 6C3HED lymphosarcoma, C3H-SX lymphoma, and L 4946 leukemia—no 2 showed the same qualitative response to all the agents by the TPCV criterion. Contrary to a commonly held belief, the Ehrlich tumor was not specifically more sensitive than all of the other tumors to any agent tested. The Sarcoma 180 ascites, although a relatively over-all insensitive tumor, was the most sensitive to both azaserine and 6-diazo-5-oxonorleucine (DON)—agents that were detected originally on an S 180 screen. Of the lymphoma-type tumors, the leukemia L 4946 was most resistant to treatment but, as a group, these tumors were most similar among themselves and most sensitive to a majority of the positive agents. These included TEM and Thio-TEPA, among the alkylating agents; Daraprim and triazines, among the antifolic agents; 8-azaguanine, 6-thioguanine, and purine, among the purine analogues; hydrocortisone and 9-α-fluorohydrocortisone, among the hormones; and actinomycin D, puromycin, fumagillin, amicetin, and Acti-dione, among agents of antibiotic origin.

Of the 22 agents found to be effective against at least 1 of the 6 tumors, 4 agents—A-Methopterin, nitrogen mustard, *N*-methylformamide, and colchicine—showed a high level of carcinostatic activity against all 6 tumors. A number of metabolic analogues and antibiotics were ineffective against all tumors.

In general, the results of this study indicate that, for detection of agents of the type tested, the system of choice for greatest over-all sensitivity is the lymphoma-type ascites tumor together with the criterion of TPCV reduction.

Summary

Total packed cell volume is a quantitative measure of primary tumor growth of ascites tumors that may be considered comparable to the parameter of total weight of solid tumors. TPCV appears to vary directly as the total tumor cell count, but can be determined more easily as the product of the ascitocrit and the total ascitic fluid weight or volume.

Reduction of TPCV of ascites tumors is a criterion suitable for cancer chemotherapy screening, but must be used with a knowledge of the TPCV growth curve and the TPCV sensitivity to body weight loss, which are characteristic for each individual tumor-host system. Under routine conditions the criterion of TPCV reduction compares favorably with those of solid tumor-weight reduction and increase in survival time. However, while survival extension is less sensitive than TPCV reduction to treatment with most of the known carcinostatic agents, there also appears to be a qualitative difference between the two criteria.

These findings, together with the demonstration of different chemotherapeutic "profiles of sensitivity" for individual ascites tumors, indicate that no single neoplasm or no single criterion is adequate for an optimal ascites tumor screen.

Acknowledgments

Acknowledgment is made to Miss F. M. Costa for direct supervision of the work reported here, and to David M. Greenberg for many helpful suggestions.

Of the ascites tumors studies, the L 4946 leukemia was kindly supplied by M. Potter and L. Law of the National Cancer Institute, Bethesda, Md., and the Hepatoma 134, by M. Belkin, also of the National Cancer Institute.

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SCREENING WITH ASCITES HEPATOMA

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The outstanding feature of studies on ascites tumors is the fact that various attributes of tumors that are either masked or difficult to observe in their solid tissue can easily be demonstrated, and that the comparison of individual neoplasms can then be more or less clearly described.

In this connection, our experiences have shown that the ascites hepatoma provides the most favorable tool. This ascites tumor can be reproduced at any place. It can present a variety of tumors of common origin, and the range of the variety can be extended as widely as needed. My associates and I now keep in our laboratory 19 transplanted strains of ascites hepatoma. The oldest strain has been maintained through about 300 generations since 1951, and the youngest one, initiated 13 months ago, has passed through about 20 generations (TABLE 1).

The ascitic convertibility of solid hepatomas induced in the liver of rats by the feeding of azo dye is not of a very high percentage. In order to establish the 19 strains, we used 156 solid hepatomas and were successful in about 12 per cent. In a few instances, in the primary hepatoma animals there occurred spontaneous ascites that contained a sufficient number of free-growing cells to establish the ascitic transplant strains, as in peritonitis carcinomatosa in man. All the strains we keep were derived from separate hepatomas developed primarily in separate animals.

The difference between the two forms, ascitic and nonascitic hepatomas, is by no means an absolute one. In cases denoted solid, there actually occurs more or less accumulation of a fluid in the peritoneal cavity that can contain a few tumor cells, so few that transfer merely with the ascites does not take.

In the ascitic form, on the other hand, the increase of tumor cells suspended in the fluid is so great as to present an almost pure culture of tumor cells. Successive transplantation with the ascites is continued. There occurs a gradual difference in the grade of this ascites formation according to the strain. In some cases the ascitic growth is so rapid that animals die chiefly because of the tremendous accumulation of the ascites. However, even in these animals there is always a development of miliary tumor nodules on the peritoneal surface that infiltrate into the underlying tissues. Also, this solid tumor formation accompanying the free growth of tumor cells in the ascites differs according to strain, as well as in individual cases. Sometimes the tumor cells showing solid growth outnumber those increasing in the fluid. Therefore the difference between ascitic and nonascitic hepatomas will be evident in the process of transplantation. In the ascitic strain the tumor can be transferred exclusively by the ascites; this is not the case as regards the nonascitic.

From these observations the ascitic growth of the tumor is comparable to the dissemination of tumor cells in the blood circulation. The two methods of growth are common in that both are based on the liberation or escape of

TABLE 1

NINETEEN STRAINS OF ASCITES HEPATOMA

Strain	Initiated in	Transplantation		Survival days	
		Generations	Rate (%)	Median	Min.–Max.
AH 130	1951	290	96.7	13	5–58
AH 7974	1952	268	96.1	12	5–76
AH 601	1952	166	82.1	18	7–133
AH 602	1952	188	91.9	16	5–120
AH 66	1954	155	90.2	12	5–71
AH 63	1955	118	80.1	11	6–126
AH 149	1955	80	88.1	16	5–139
AH 39	1955	95	80.0	18	5–130
AH 49	1955	94	75.7	17	6–119
AH 99	1956	69	90.5	14	5–83
AH 66F	1956	81	90.9	8	5–40
AH 322	1957	49	60.0	15	6–73
AH 414	1957	37	81.6	19	7–98
AH 21	1957	45	77.0	9	5–30
AH 423	1957	43	50.6	31	6–105
AH 364	1957	54	92.8	11	5–70
AH 318	1957	46	77.2	10	6–55
AH 13	1957	62	94.1	7	5–24
AH 62	1957	20	84.4	18	7–49

separated cells into the fluid medium from the focus, or solid growth, where the tumor cells were initially concentrated.

On the other hand, tumor cells of the ascites hepatoma, increasing while suspended in the fluid, also keep their epithelial nature. Unlike other ascites tumors generally known, they are not individually separated, but form bonds of cells and further clusters of various sizes that we call islands.[1, 2] It is notable that each strain has its own pattern of composition for its island, that is, its own ascitic picture in which the size, shape, solidity of cell-bonding, and other factors vary according to the strain. The islands remain unchanged through serial transfers and so differ from each other that each strain can easily be identified under a microscope. This observation was the starting point of our researches in the individuality complex of the ascites hepatoma.[2]

In this connection I propose to discuss: (1) dissemination of tumor cells in animals with ascites tumor; and (2) individual differences of hepatomas in their responses to chemical compounds.

Dissemination of Tumor Cells

The formation of metastasis in animals bearing transplanted ascites tumors is a rare occurrence, probably owing to the short survival time of these animals.[3] However, in animals surviving as long as one to two months under experimental chemotherapy treatment, metastatic growths occurred often, and their sites were sometimes unusual, as on the cheek or the skull. This experience evoked interest as to how early after the inoculation of tumors, and how frequently, the dissemination of tumor cells would take place in animals into which they were transplanted.

TABLE 2

EARLIEST DETECTION OF TUMOR CELLS* IN SMEARS OF HEART BLOOD

Strains	AH 13	AH 66F	AH 130	AH 7974	AH 49
Days	3	3	6	7	11

* Collected by a modification of Sandberg's method.

TABLE 3

RESULTS OF INTRAPERITONEAL TRANSPLANTATION, USING THE HEART BLOOD OF FOUR-DAY-OLD ASCITES HEPATOMA ANIMALS

Results	Strains		
+	AH 130	7974	66
	66F	149	39
	49	13	21
–	AH 601	602	63
	99	322	414
	423	364	318
	62		

The result of a preliminary microscopic search for the presence of tumor cells in the circulating blood of ascites hepatoma animals is shown in TABLE 2. Further assay of the blood by its transplantation into normal rats proved a much earlier dissemination. In about one half of four-day-old tumor animals examined, the heart blood contained a sufficient number of tumor cells for positive transplantation (TABLE 3). Further experiments in transplantation, using blood as well as the minced material of the lung tissue, demonstrated that dissemination occurred as early as one day after inoculation with the tumor (TABLES 4 and 5).

Experiments are now in progress to inoculate subcutaneous tissue with ascitic hepatoma cells. Results thus far obtained indicate that there is no essential difference in this respect between intraperitoneal and subcutaneous inoculations.

A systematically designed experiment concerning the dissemination of tumor cells as well as their inhibition by chemical means, using rats' ascites hepatoma, is being carried out by Tosuke Kurata and others in our laboratory.[4] Sato is also working with me in this investigation, using the ascites hepatomas of mice. It was Sato who established, in the National Cancer Institute, Bethesda, Md., these transplant strains by ascitic conversion of spontaneous hepatomas in mice supplied there by H. B. Andervont. Sato maintains three strains in Japan.

These ascites hepatomas, inoculated into subcutaneous tissue, give rise to uniformly growing solid tumors at the site of inoculation which never show a spontaneous regression. Surgical removal of such tumors caused a slight prolongation of life of the animals, but all of them died on regrowth of the tumor, which grew either at the initial site or in the lymph nodes of the axillary and/or inguinal regions.

TABLE 4

EARLIEST APPEARANCE OF TUMOR CELLS* IN HEART BLOOD AFTER TRANSPLANTATION OF ASCITES HEPATOMAS

Strains	AH 13	AH 66F	AH 130	AH 7974	AH 49
Days	1	1	3	4	4

* After intraperitoneal transplantation with heart blood of the tumor-bearing donors at various days after transplantation.

TABLE 5

DETECTION OF TUMOR CELLS BY INTRAPERITONEAL TRANSPLANTATION WITH THE HEART BLOOD AND LUNGS OF THE TUMOR-BEARING DONORS

Strains	Materials obtained, days after transpl.	No. of donors	Positive			Negative
			Both in the blood and lungs	Blood only	Lungs only	
AH 13	1	2	1	0	1	0
AH 66F	1	2	2	0	0	0
	2	2	2	0	0	0
AH 130	2	2	0	0	0	2
	3	2	1	0	0	1
	4	2	2	0	0	0
	6	2	2	0	0	0
AH 7974	2	2	0	0	0	2
	3	2	0	0	0	2
	4	2	1	0	1	0
AH 39	4	3	2	0	0	1
AH 601	4	3	0	0	1	2
AH 602	4	3	0	0	0	3
AH 318	4	3	0	0	0	3

An attempt was made to prevent these regrowths and metastases by chemotherapy with HN2 N-oxide. Two weeks after the initial inoculation, tumors were surgically removed and treatment was begun by intraperitoneal administration of the compound for 10 to 15 successive days.

As summarized in TABLE 6, 9 of 72 animals that received chemotherapy survived a period of observation of 100 days without showing any sign of regrowth and metastatic formation. This result may not be very striking but, taking into account the fact that no animal has ever been cured by mere excision, the 9 cases were considered as cured by the chemotherapy that followed the operation.

On the other hand, experiments in transplantation with materials from the lung and lymph nodes of tumorous animals demonstrated that dissemination of cells into these distant organs had already occurred within five days

TABLE 6

SURVIVAL TIME OF THE MICE INJECTED WITH NITROMIN FOLLOWING EXCISION* OF SUBCUTANEOUS TUMORS OF ASCITES HEPATOMAS

Tumors	MH 134			MH 129F			MH 129P		
	Control	Operation only	Operation + chemotherapy	Control	Operation only	Operation + chemotherapy	Control	Operation only	Operation + chemotherapy
Total No. of animals	11	24	24	8	26	25	5	31	23
Survived more than 100 days	0	0	4	0	0	5	0	0	0

* Operation was performed two weeks after the inoculation.

TABLE 7

DETECTION OF TUMOR CELLS IN THE LYMPH NODES AND LUNGS OF MICE WITH SUBCUTANEOUS TUMORS

(C3H/HeN Mouse Ascites Hepatomas)

Inoculum obtained from	Growth of tumor cells in ascites of the recipients		
	Group I* (5 days)	Group II* (10 days)	Group III* (15 days)
Lymph nodes	2/7†	12/18	18/24
Lungs	2/7	7/18	17/24

* Transplanted with inoculum obtained from the donors bearing 5-, 10-, and 15-day-old subcutaneous tumors, respectively.

† Number of the "takes"/number of the recipients.

(TABLE 7). From this it became clear that chemotherapy begun two weeks postoperation would be too late.

Further experiments are being carried out in which the chemotherapy following the operation is begun on earlier days.

In this connection recent studies on detecting cancer cells in the venous blood draining tumor areas are of special interest. Engell's study[5] in this field resulted in the successful detection of cancer cells in 59 per cent of 107 cases examined without showing any significant difference between detection before and after the surgical manipulation of tumors. Tumor cells were found with equal frequency in patients who developed metastases, or recurrence, as in those who survived without evidence of metastases or recurrence. Sandberg and Moore confirmed Engell's observations by obtaining almost the same results in their own clinical studies.[6]

Individual Differences of Hepatomas in Their Responses to Chemical Compounds

TABLE 8 outlines our recent studies on the different responses of various ascites hepatomas to chemical treatments. This table shows that in some strains that proved resistant to treatment with one half the maximum tolerance dose, cytological examinations could also reveal the characteristic mitotic effect of

TABLE 8
NITROMIN

Strains	Half of M.T.D.	
	Prolongation of life*	Cytological effect
AH 66F	+	+
AH 13	+	+
AH 130	+	+
AH 99	+	+
AH 63	+	+
AH 601	+	+
AH 414	+	+
AH 49	−	+
AH 62	−	+
AH 39	+	+
AH 602	−	+
AH 21	−	+
AH 318	−	+
AH 322	−	−
AH 149	−	−
AH 7974	−	−
AH 423	−	−
AH 364	−	+
AH 66	−	−

Symbol: + means that more than one half of all treated animals survived more than twice as long as control animals.

* Half dose of M.T.D. was injected into 4-day-old tumor animals.

HN2, although slight in degree. Attempts were made to obtain more accurate information by means of the so-called *in vitro-in vivo* test initiated by Hiroshi Sato.

The procedure for the test is as follows. To 1.0 cc. of tumor ascites tapped from 4-day-old tumor animals, containing about 100 million cells, is added, in the test tube, an amount of Nitromin dissolved in 1.0 cc. saline solution. After 30 min. incubation at 37° C. the entire content of the test tube is inoculated into the peritoneal cavity of a normal animal whose tumor-ascites is examined for the characteristic cytological changes every 24 hours after the inoculation. By gradually reducing the amount of the substance given *in vitro*, the minimum effective dose for inducing the cytological changes is determined. The result is indicated in the third column of TABLE 9. The difference in sensitivity of various tumors to a compound was thus demonstrated, not only for each tumor as a whole, but on a cellular basis, much magnified.

The different behavior of various hepatomas against chemical treatment was not a peculiar case for HN2 N-oxide. Other compounds belonging to alkylating agents, such as TEM, Thio-TEPA, and Myleran, also showed similar relations to Nitromin. An example with Thio-TEPA is shown in TABLE 10.

As to compounds other than alkylating agents it is difficult, in general, to demonstrate their effect on ascites tumors. This has long been a problem in our screening work with ascites tumors, but the result of our recent experiment with 6-mercaptopurine is interesting. It was successfully demonstrated that obvious differences existed also among various ascites hepatomas in response

TABLE 9
NITROMIN

Strains	Half of M.T.D.		M.E.D. *in vitro* (γ/cc.)
	Prolongation of life*	Cytological effect	
AH 130	+	+	0.1
AH 7974	−	−	5.0
AH 601	+	+	5.0
AH 602	−	+	0.5
AH 66	−	−	5.0
AH 63	+	+	5.0
AH 149	−	−	50.0
AH 39	+	+	1.0
AH 49	−	+	10.0
AH 27	−	−	10.0
AH 99	+	+	0.1
AH 66F	+	+	0.1
AH 322	−	−	50.0
AH 414	+	+	5.0
AH 21	−	+	5.0
AH 423	−	−	10.0
AH 364	−	+	5.0
AH 318	−	+	10.0
AH 13	+	+	0.1
AH 62	−	+	50.0

Symbol: + means that more than one half of all treated animals survived more than twice as long as control animals.

* Half dose of M.T.D. was injected into 4-day-old tumor animals.

TABLE 10
THIO-TEPA

Strains	Half of M.T.D.	
	Prolongation of life	Cytological effect
AH 66F	+	+
AH 13	−	+
AH 130	−	+
AH 99	−	+
AH 63	−	−
AH 601	−	−
AH 414	−	−
AH 49	−	−
AH 62	−	−
AH 39	−	−
AH 602	−	−
AH 21	−	−
AH 318	−	−
AH 322	−	−
AH 149	−	−
AH 7974	−	−
AH 423	−	−
AH 364	−	−
AH 66	−	−

TABLE 11
6-MERCAPTOPURINE

Strains	Half of M.T.D.	
	Prolongation of life	Cytological effect
AH 66F	−	−
AH 13	+	+
AH 130	−	−
AH 99	+	+
AH 63	+	+
AH 601	−	−
AH 414	−	+
AH 49	−	−
AH 62	−	−
AH 39	−	−
AH 602	−	−
AH 21	−	−
AH 318	−	−
AH 322	−	−
AH 149	−	−
AH 7974	−	−
AH 423	−	−
AH 364	−	−
AH 66	−	−

to this compound (TABLE 11). The compound proved to be effective in three of nineteen strains. Not only the retardation of growth of the tumors as a whole, but also its cytological effect, was convincing. This finding encouraged us to extend the experiments to other kinds of antimetabolites, such as derivatives of antifolic acids, in the expectation of finding some strains of ascites hepatoma especially sensitive to antimetabolites. For this purpose we plan to enlarge the variety of strains of the ascites hepatoma.

Summary

Considerations on screening procedures from the described observations may be summarized as follows:

(1) From information obtained from both experimental and clinical observations, the dissemination of cancer cells may properly be regarded as a condition of equal pathological significance to localized tumor growths. It is true that localized tumors are the chief cause of serious clinical symptoms, as well as of complaints of the patients, but it is also true that they alone by no means represent the whole of the condition The causative agent of the cancer disease, the cancer cell, is always, in my view, spreading by means of the circulation throughout the body of the patient. From this point of view, especially from the standpoint of treating cancer, it may not be too much of an exaggeration to compare the disease with the septicemic condition of an infectious disease, even though the malignant growth gives it a contrary appearance.

In the treatment of cancer further attention should be paid to this point, which reaches beyond the surgical and radiation approaches. Chemotherapy finds its place here, and the ascites tumor may serve as a useful tool for experimental work in this direction.

At the meetings of the International Symposium on Cancer Chemotherapy held in Tokyo in October 1957, "there was agreement that against the general spread of cancer with the formation of metastasis in other parts of the body, beyond the reach of surgery and radiation, chemotherapy offers the greater hope," to use the phraseology of Murray J. Shear in summarizing the symposium.

(2) The fact that various strains of the ascites hepatoma responded in different ways to one and the same compound runs contrary to expectations that one might have for a compound of over-all effect on neoplastic growth in general. However, this fact is at the same time encouraging, because it demonstrates that some ascites hepatomas are especially sensitive to certain compounds. In other words, it suggests that there are compounds that have a special effect upon definite groups of cancers. This may provide a basis for the attempt to establish a tumor "spectrum" consisting of ascites hepatomas of different sensitivities to the known anticancerous compounds; the designed spectrum may involve the ascites hepatomas that are especially sensitive to alkylating agents and to antimetabolites and, finally, those that are resistant to all the known anticancer agents.

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Part IV. Lymphomas

MORPHOLOGY AND PATHOLOGY OF RETICULAR NEOPLASMS IN THE MOUSE: RELATIONSHIP TO MAN

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Unfortunately, one can never begin a discussion of this group of neoplasms without becoming involved in the meaning of terms. In referring to neoplasms originating from cells of the blood-forming organs or the reticuloendothelial system in the mouse, it is customary to group all of them under the term "leukemia."[1] I prefer the term "reticular neoplasms," although I readily admit that there are valid objections to this usage.[2] I have also adopted, on what I believe is good authority, "reticulum cell" as a term inclusive of, first, the solitary fixed cells forming the cellular elements of the main reticular organs and tissues; second, the histiocytes; and third, the monocytes.[3]

In a paper published 23 years ago, Furth *et al.*[4] pointed out the resemblance between lymphocytic leukemia in the mouse and leukemia in man. In this same paper the authors also noted that each transplanted leukemia line in the mouse had its own individual characteristics. The conclusions reached in this paper hold true today, although many more leukemia lines now have been described and new methods for study are available. The method of transforming a reticular neoplasm into an ascites form and examining the cells under the phase microscope has given more exact information regarding the morphology of the cells of these tumors than we could obtain previously. Let me emphasize that, although for convenience I plan to discuss these neoplasms as if they belonged to well-defined groups, one should always think of a transplanted line as something unique, exhibiting its own peculiar biological behavior and morphology. The pathologist is no more able to classify neoplasms and fit each one into a rigidly defined category than the anthropologist is able to differentiate sharply the races of mankind. We could not expect, therefore, that any reticular neoplasm in the mouse would exactly parallel a human neoplasm, even though the cell of origin is the same. General resemblances are striking, however, and a close relationship certainly exists between these disease processes in the two species.

In both man and mouse it is practicable to separate reticular neoplasms into various groups, depending upon the normal cell they most closely resemble (TABLE 1). As indicated in TABLE 1, the neoplasms derived from the lymphocyte and granulocyte have a close resemblance in man and in the mouse. The neoplasms in the mouse derived from the reticulum cell show the least resemblance to neoplasms of this derivation in man. Particular plasma cell neoplasms have shown some striking parallels in the two species. The mast cell neoplasm in man occurs too infrequently to allow for many comparisons, but the individual neoplastic cells are similar.

TABLE 1
COMPARISON OF RETICULAR NEOPLASMS IN MAN AND MOUSE

Neoplasm in mouse	Normal cell	Neoplasm in man
+ Lymphocytic neoplasm, localized + Lymphocytic neoplasm, generalized	Lymphocyte	Lymphosarcoma Lymphocytic leukemia
+ Granulocytic neoplasm	Granulocyte	Granulocytic leukemia
– Reticulum cell sarcoma, type A ? Hodgkin's-like lesion, type B	Reticulum cell	Reticulum cell sarcoma Hodgkin's disease
+ Plasma cell neoplasm	Plasma cell	Multiple myeloma
+ Mast cell neoplasm	Tissue mast cell	Tissue mast cell leukemia

Symbols: + = close resemblance between tumors from the two species; – = little resemblance to any neoplasms of man; ? = uncertainty regarding resemblance or relationship to human neoplasm.

Lymphocytic Neoplasm

The lymphocytic neoplasms in the two species are remarkably similar. FIGURE 1 shows lymphocytic leukemia cells infiltrating between kidney tubules. Notice the similarity in individual cells in the two neoplasms. Lymphocytic leukemia has been the most frequently studied and the most widely used for chemotherapeutic trials in the mouse; it appears in high incidence and at an early age in certain inbred strains, is readily transplanted, and usually kills the mouse in about 10 days, thus allowing a quick and positive answer in screening procedures.

Other forms of reticular neoplasms have been less frequently studied and used for chemotherapy. One reason is that these neoplasms are relatively rare and difficult to obtain. The growth rate in early generations is not so rapid as with lymphocytic tumors, and the survival times of untreated mice bearing the tumors may vary by many days or weeks. However, many different forms of reticular neoplasms are now becoming available as transplantable lines often obtainable in an ascites form, so that the number of tumor cells injected can be accurately measured.

Granulocytic Neoplasm

For comparison of the granulocytic neoplasms in the two species, FIGURE 2 shows a blood smear from a human case of acute granulocytic leukemia and cells from the ascites fluid of a granulocytic neoplasm in the mouse. In both neoplasms the cells are undifferentiated, but granules can be seen in the cells in the mouse. This neoplasm requires from 14 to 16 days to kill the host, in contrast to a killing time of 8 to 12 days with some lymphocytic leukemias.

Reticulum Cell Sarcoma, Type A

The reticulum cell sarcoma, type A, in the mouse also is termed, appropriately, a histiocytoma, or monocytic neoplasm. It is an almost esthetically

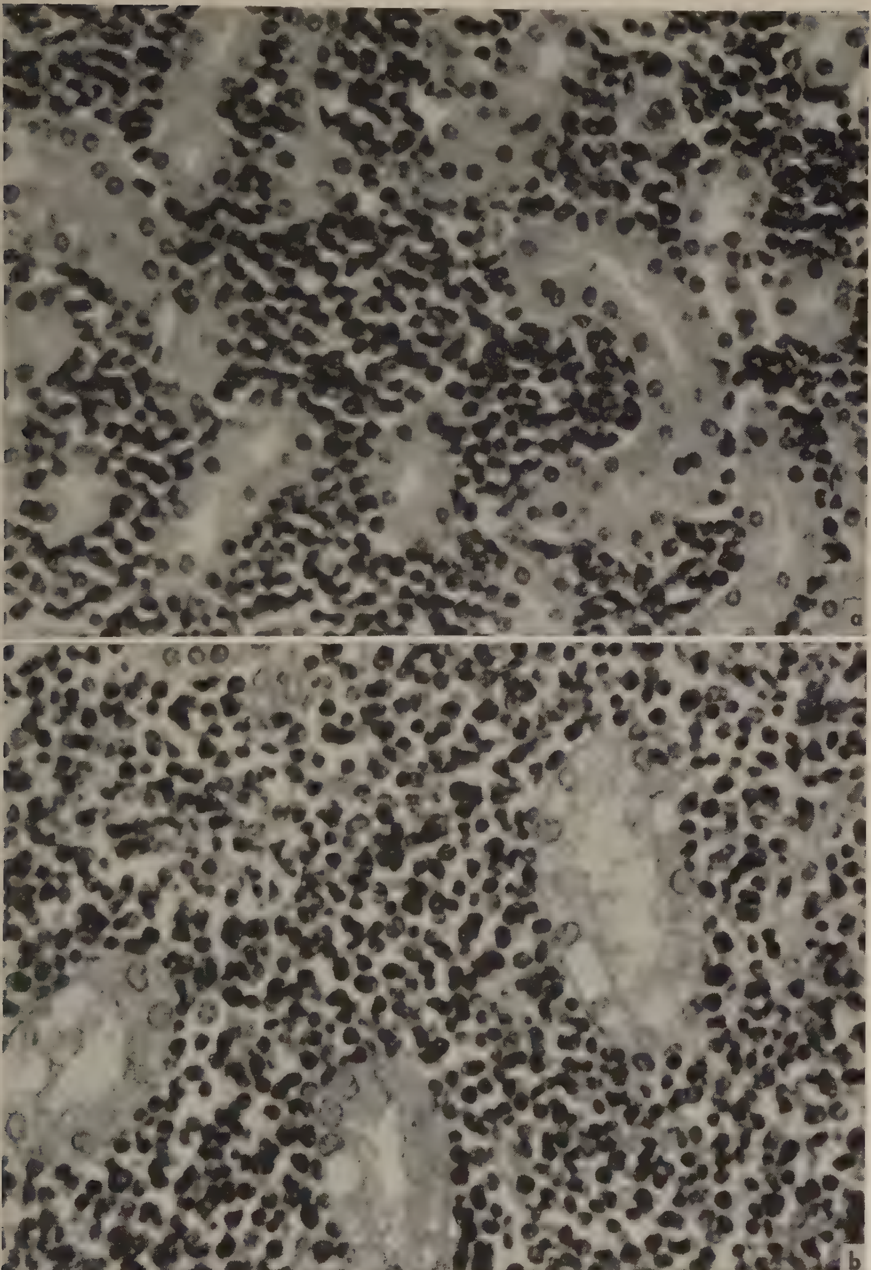

FIGURE 1. Lymphocytic leukemia, showing neoplastic cells infiltrating between kidney tubules: (a) mouse, (b) man. Note the similarity in cell details. Hematoxylin and eosin. ×420.

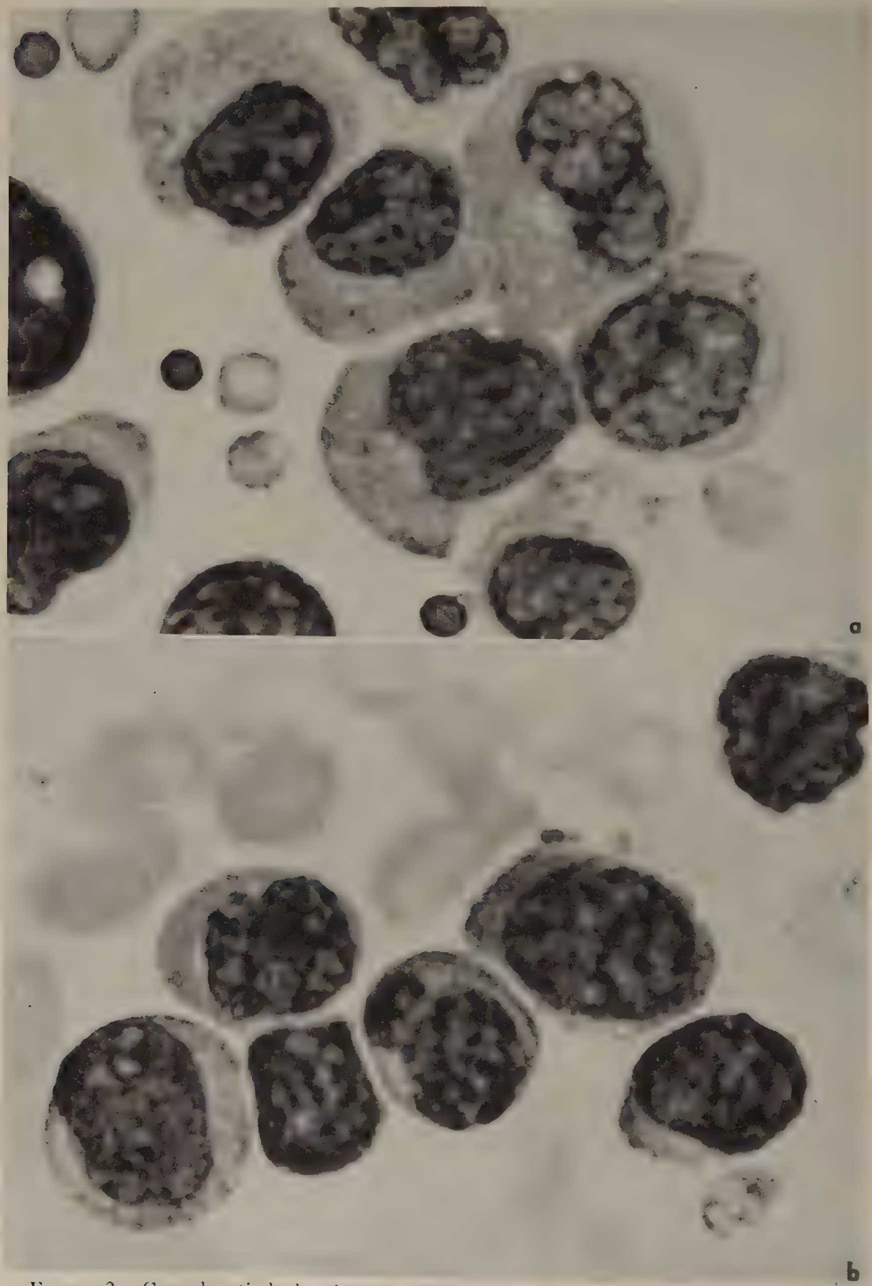

FIGURE 2. Granulocytic leukemia: (a) cells from ascites tumor fluid in mouse, (b) human blood smear. The cells from each are undifferentiated, but the ascites cells contain granules. Wright's stain. ×420.

pleasing subject for study, because it has a unity or wholeness that the Greeks considered requisite to any work of art. The neoplasm clearly reproduces many characteristics of the histiocyte or monocyte from which it is derived. It appears to be much better differentiated than the corresponding human neoplasm (FIGURE 3). The shape of individual cells varies from a long spindle arranged in bundles like fibroblasts to a large round discrete cell lying free. The same variations in shape appear in normal histiocytes and monocytes. The cells usually show phagocytic capacity, and they often produce hematoidin crystals from extravasated erythrocytes, another indication of physiological function. As a rule, only a small local tumor develops from subcutaneous transplantation, but the tumor grows diffusely in the liver. These tumors usually arise in older mice, and they have a slow growth rate. Some have required 6 months or longer to kill the host after transplantation. They are relatively common tumors in the mouse, while in man they are rare. Furthermore, the human tumors fail to reproduce so clearly the characteristics of the histiocyte.

Reticulum Cell Sarcoma, Type B (Hodgkin's-like Lesion)

A lesion has been found in old mice that has some resemblance to Hodgkin's disease in man (FIGURE 4). We have called it reticulum cell neoplasm, type B, or the Hodgkin's-like lesion. It usually arises in mice past 1 year of age, and it grows slowly. Microscopically, the tissue consists of a large, pale, reticulum type cell of variable size, sometimes forming giant cells, but I have never found cells that accurately mimic the Reed-Sternberg cell. Plasma cells and lymphocytes usually are mingled with the large pale cells. The fibrosis and eosinophilia found in the human disease are never prominent. The Hodgkin's-like process in the mouse does not often exhibit a well-defined, consistent picture in all the different organs or tissues involved. The lesions of some lymph nodes, for example, may resemble lymphocytic leukemia, and the microscopic picture in other nodes may resemble a reticulum cell sarcoma. The Hodgkin's-like process sometimes suggests an infectious granuloma, with Langhans-type giant cells scattered in the tissue.

Much more study will be required before I can speak with any assurance of this lesion in the mouse. Although a few of these lesions had been successfully transplanted previously, Michael Potter recently has succeeded in transplanting several, which indicates that at least some are truly neoplastic. In contrast to the histiocytomas, they do not produce good ascites tumors, but several free cells in the peritoneal fluid have been obtained for phase-microscope studies and stained smears. We hope to have a much better understanding of this lesion when the transplanted growths have been followed closely. Attempts to understand and define the disease in the mouse are as baffling as attempts to characterize Hodgkin's disease in man. Indeed, it may be said that the greatest similarity between this process in the mouse and Hodgkin's disease is the perplexity that each causes the pathologist.

Plasma Cell Neoplasm

The plasma cell neoplasms in the mouse have only recently become available in transplant. We are now carrying three lines. Cells from two of the lines

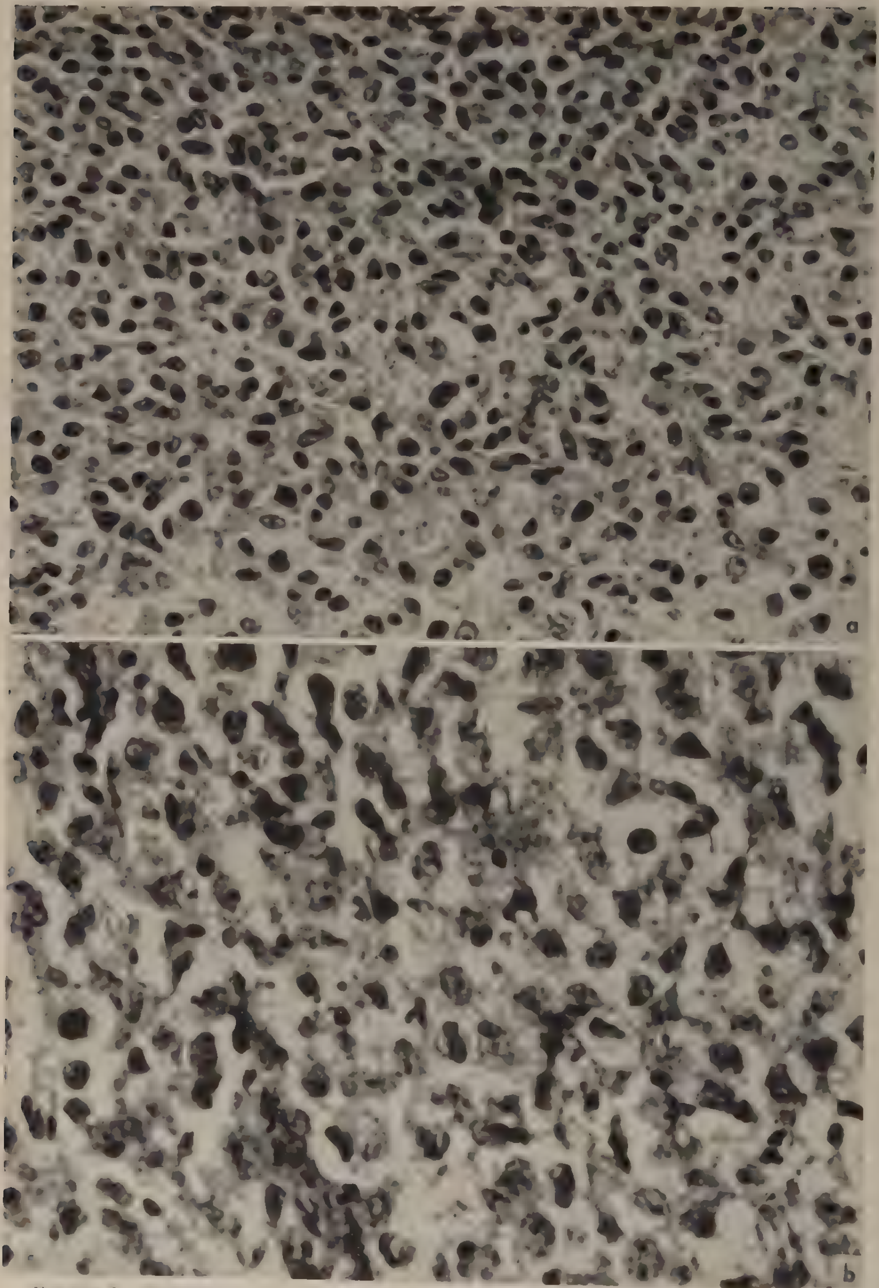

FIGURE 3. Reticulum cell sarcoma: (a) neoplasm in mouse, composed of well-differentiated histiocytic cells (type A); (b) human neoplasm, composed of large, pale staining, undifferentiated cells. Hematoxylin and eosin. ×420.

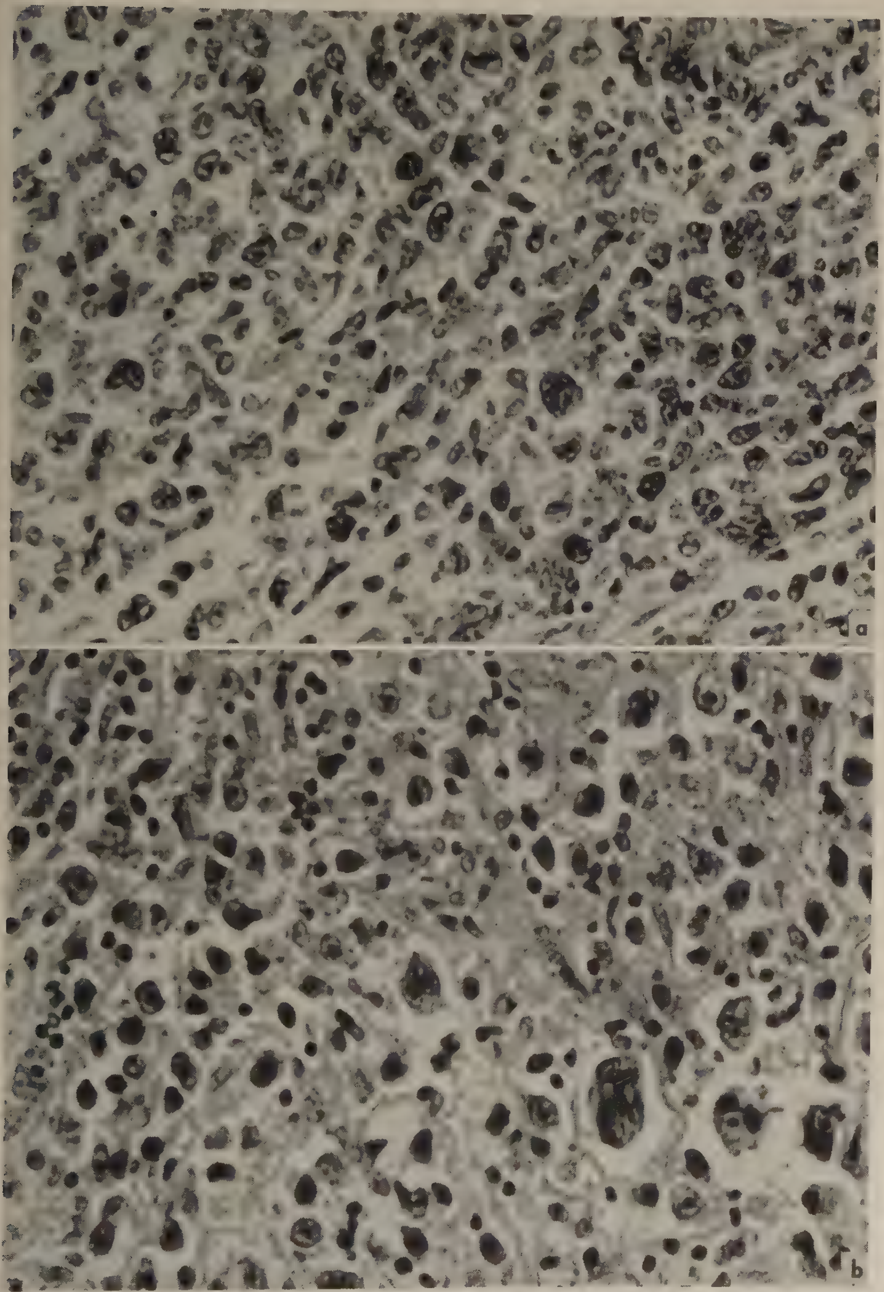

FIGURE 4. (a) Reticulum cell neoplasm, type B (Hodgkin's like lesion) in mouse; (b) Hodgkin's disease in man. The lesion in the mouse is composed of large reticulum cells, which may be multinucleated. Human disease shows fibrosis and Reed-Sternberg cells. Hematoxylin and eosin. ×420.

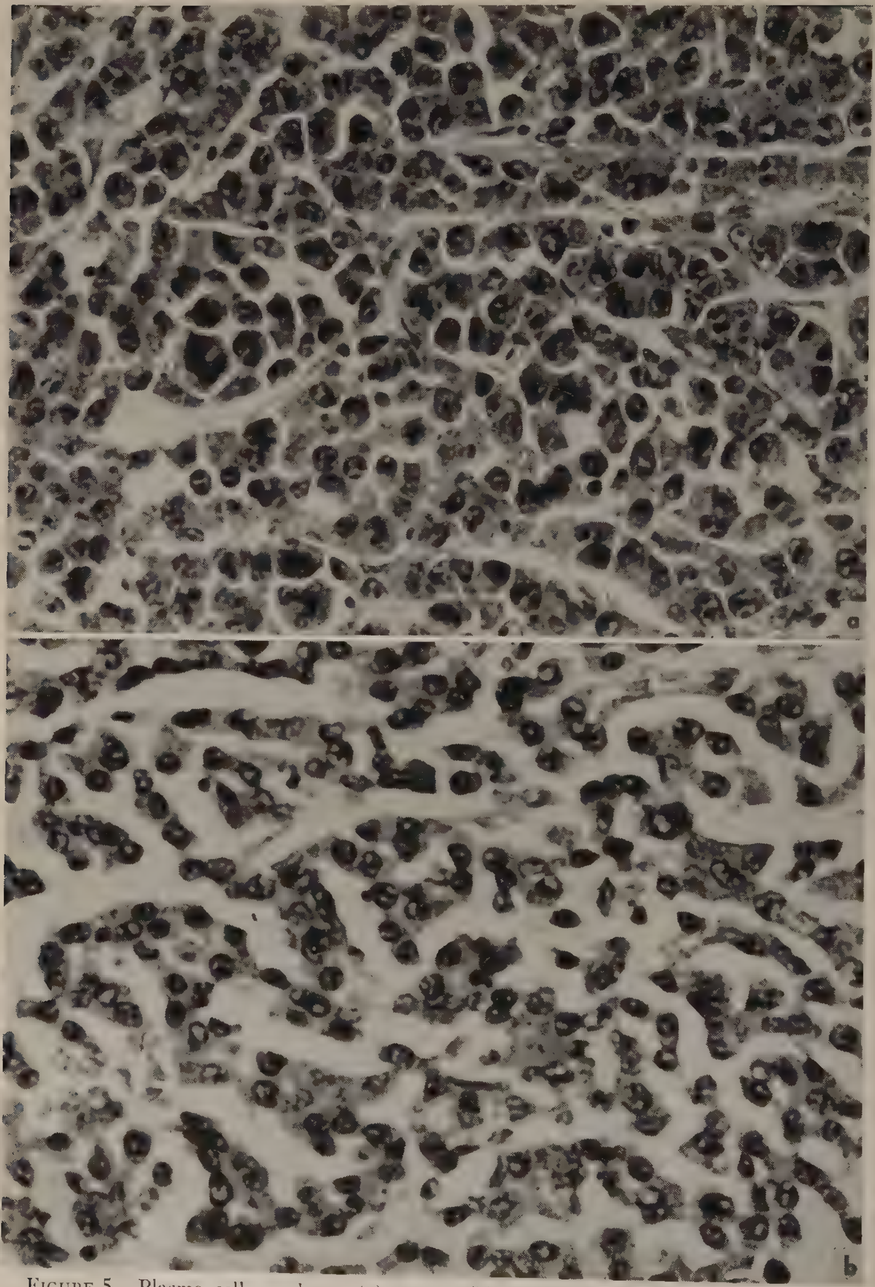

Figure 5. Plasma cell neoplasm: (a) mouse, (b) multiple myeloma in man. The cells in the mouse resemble normal plasma cells in nuclear structure and clear perinuclear area. Hematoxylin and eosin. ×420.

closely resemble normal plasma cells, and they also show a close resemblance to the cells of multiple myeloma in man (FIGURE 5). The most remarkable resemblance, however, is the biochemical change in the blood plasma of the tumor-bearing hosts. Abnormal proteins are found in both man and mouse. Our tumors originated at the ileocecal area in strain C3H mice.[5] Other primary plasma cell neoplasms have been widely disseminated, resembling a leukemia.[6] One transplanted plasma cell tumor of the mouse has caused punched-out areas in the bone, such as appear in multiple myeloma.[7] The three plasma cell tumors we have studied in the mouse all originated from the same cell type, yet each has shown characteristics peculiar to itself. These differences admirably illustrate the individuality of tumor lines.

Mast Cell Neoplasm

Transplantable mast cell neoplasms in the mouse have recently become available. Potter and I[8] are carrying one that grows rapidly and contains relatively few metachromatic granules when compared with a mast cell tumor carried by Furth.[9] Furth's tumor grows more slowly, and the cells are better differentiated. Mast cell neoplasms now have been reported in man, but they are so rare that opportunity for chemotherapy trial, even if it could be achieved, would be rare in a human patient.[10] Our transplantable mast cell tumor has proved valuable in studying the chemical components of mast cells. The tumors have been found to contain large amounts of serotonin, histamine,[11] and heparin (Korn, E. D., unpublished data).

This completes the review of the principal transplantable reticular neoplasms in the mouse. Some of these are closely analogous to neoplastic diseases in man and might be used for testing drugs proposed for cancer chemotherapy. Because of the individuality of each transplanted line, no predictions can be made as to how a particular line will react to a particular drug, and generalizations should be avoided. The fact that a drug succeeds or fails with one neoplasm may not accurately foretell the effect it will have on another.

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Discussion of the Paper

Jacob Furth (*The Children's Cancer Research Foundation, Boston, Mass.*): Thelma Dunn's article* on neoplasms, leukemias, and related conditions in mice is an excellent work. Very few pathologists in the United States are familiar with neoplasia and leukemia in small laboratory animals, and her report has been a source of information for all of those who wish to learn about the leukemias of mice.

I consider it very unfortunate, however, that she introduced the term "reticular" as a common denominator of leukemialike diseases. This term has been used by many workers to cover many things that are not well understood.

Dunn: I think my use of the term "reticular" was a result of pure laziness. I was so weary of writing hemopoietic and blood-forming organs and lymphocytic that I sought one word that might include them all. I admit it is not the most satisfactory term. I wish someone would suggest a better word; I should be glad to use it.

Furth: The term "hemoblastosis," used in France, seems to me to be preferable.

Question: From Dunn I should like to know something of the distribution of the normal mast cells in the presence of a tranplantable mast cell tumor. Do the granules persist in as great a number, or is there any variation in the activity of the normal mast cell in the presence of these?

Dunn: I have been unable to see any effect on the normal mast cell.

Michael Potter (*National Cancer Institute, Public Health Service, Bethesda, Md.*): This is no place for a pathological wrangle, but I should like to add one pertinent remark. I do not think many pathologists (I am not a pathologist) would have much difficulty in diagnosing a lesion that resembled normal tissue; that is, for example, the lymphocytic neoplasm that looks like the lymphocyte, or the mast cell neoplasm that looks like a mast cell. The real difficulty in diagnosing lesions of the reticular system stems from the fact that there are many lesions that originate in cells that have not progressed to this stage of differentiation. It is here that the battle as to which is which begins. A tissue consisting of functional cells and also of reserve cells has been described. Here we have a system that Dunn refers to as a reticular system, and others as the hematopoietic system, which has essentially seven differentiative cell types: the red blood cell, the megakaryocyte, the lymphocyte, the plasma cell, the monocyte, the mast cell, and the granulocyte. These are functional cells.

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* Dunn, T. B. 1954. Normal and pathologic anatomy of the reticular tissue in laboratory mice, with a classification and discussion of neoplasms. J. Natl. Cancer Inst. **14:** 1281–1433.

The cells that lie behind these are the reserve cells, and this is where we encounter our difficulty. It is very important that we reckon with this group, however, because there are diseases—formidable diseases—that originate in reserve cells, and some of these may be Hodgkin's disease or reticulum cell sarcoma.

QUESTION: What does Furth mean by a conditional neoplasm with respect to the *in vitro* sarcoma?

FURTH: A conditioned neoplasm, in contrast to autonomous neoplasia, is not transplantable to a normal host. It will grow when the stimulating factors are in excess or when restraining factors are deficient.

VARIATION IN RESISTANCE PATTERNS IN DIFFERENT NEOPLASMS

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Studies with experimental mouse neoplasms have shown that the phenomenon of drug resistance imposes serious limitations upon the effectiveness of antimetabolites as anticancer agents.[1] Although this phenomenon presents only one of the many reasons why antimetabolites may fail to cure transplantable neoplasms, further understanding of this property may lead to specific methods of obviating resistance.

The study of resistance in mouse neoplasms is still in its infancy. The largest accumulation of information concerning drug resistance in experimental neoplasms has been with the antimetabolites. A paucity of information exists concerning such other classes of anticancer agents as the alkylating agents and hormones. It may be important, in all extensive chemotherapeutic studies on specific compounds, to know whether drug resistance as a phenomenon is a limiting factor in attaining maximal antitumor effectiveness.

The present observations have been selected from three neoplasms in which inhibition of growth has been demonstrated by an antimetabolite. These observations demonstrate that drug resistance may develop by different biological mechanisms in a single neoplasm. The three neoplasms have been selected from various transplantable mouse ascitic neoplasms of reticular origin. The three neoplastic cell types selected were a plasma cell neoplasm, a mast cell neoplasm, and a generalized lymphocytic neoplasm.

Materials and Methods

Plasma cell neoplasm 70429. This is a diploid neoplasm that developed in the ileocecal region of a C3H/He mouse injected with methylcholanthrene. It is not associated with abnormal serum globulin production, as are certain other plasma cell neoplasms carried in this laboratory.[2]

The origin and conversion of this neoplasm to the ascitic form have been described previously.[3, 4] The origin of the two azaserine-resistant sublines, AZR-I and the 173DR, was described also in the previous publication. These lines have been maintained in serial transplant in C3H mice of various sublines maintained at the National Cancer Institute: these include C3Hf/Lw, C3He/De, and C3H/He N. They were carried as ascitic neoplasms in each case.

Mast cell neoplasm P 815. The origin of this neoplasm has been described by Dunn and Potter,[5] and the values of serotonin and histamine found in it have been reported in a subsequent publication by Sjoerdsma *et al.*[6] The neoplasm was converted to an ascitic neoplasm at the fourth transfer generation and has since been maintained in the ascitic form. The transfer was made in (BALB/c × DBA/2)F_1 hybrids. All studies reported here were carried out in these hybrids.

Lymphocytic neoplasm P 288. The origin of this neoplasm has not been described previously. A DBA/2 male mouse was given 20 skin paintings of 0.2 per cent 3-methylcholanthrene in ether 3 times weekly. Eight different painting sites were employed to reduce the incidence of skin neoplasia. At 5 months of age a large mass was noted in the left axillary region. The animal was sacrificed, and a mass of tissue with lymphoid appearance, apparently arising from an axillary lymph node, was found. The thymus and spleen were slightly enlarged. All of the peripheral lymph nodes were enlarged. Microscopically the process consisted of a generalized lymphocytic neoplasm widely disseminated to the liver, spleen, kidney, and lymph nodes. The sections were reviewed by Thelma Dunn, who described the cell as lymphoid in character, unusually large, with a rosette-shaped nucleus. The neoplasm was transplanted intraperitoneally, and it became ascitic in the first transfer generation. Ascitic smears revealed that the chief cell was a large lymphoid cell, with scant cytoplasm and a very striking rosette-shaped nucleus The neoplasm has been maintained as an ascitic neoplasm and has been serially transferred in (BALB/c × DBA/2)F_1 hybrids. An inoculation of 1 million ascitic tumor cells usually kills the host in 9 to 11 days. The ascites has been grossly bloody consistently. Aceto-orcein squash preparations were made to determine the chromosome number at the twelfth transfer generation. Both diploid and near-tetraploid cells were found. Owing to difficulties in spreading the chromosomes, exact appraisal was not made at this time. Aceto-orcein smears were made by T. S. Hauschka at the forty-ninth transfer generation, at which time the neoplasm was found to be purely in the tetraploid range.[7] It is not possible to state that this neoplasm arose as a polyploid neoplasm. From the large size of the cells, it is probable that at the time of origin the population was a mixture of diploid and polyploidal elements. By the forty-ninth transfer generation the near-tetraploid cell dominated the population. Studies of response of this ascitic neoplasm to A-Methopterin were begun at the fourth transfer generation. At the present time this neoplasm is in its one hundred and twentieth transfer generation.

Antimetabolites. The antimetabolites employed in this study have been azaserine* (*O*-diazoacetyl-L-serine), DON* (6-diazo-5-oxo-L-norleucine), and A-Methopterin† (Methotrexate).

The antimetabolites were administered subcutaneously in the posterior interscapular region. The dosage and frequency of treatments are as follows: azaserine, 5 mg./kg., and DON, 0.125 mg./kg., 5 to 6 times weekly; and A-Methopterin, 3 mg./kg. three times weekly, given every other day. The azaserine and DON treatment schedules were well tolerated by the mice. A-Methopterin produced toxicity beginning in the third week, as described below. Azaserine and DON were prepared in phosphate buffer and were frozen at −10° C. when not in use.

Cell counting. The cell-counting procedures have been described previously.

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* Supplied by Parke, Davis & Company, Detroit, Mich.

† Supplied by the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.

All dilutions were made in chilled Locke's solution. Serial tenfold dilutions were made with separate serologic pipettes.

Percentage increase in survival. This is determined by subtracting the average survival time in days of the controls from the average survival time of the treated mice, and dividing by the average survival time of the controls.

Mice. The mice employed in these experiments were housed in plastic cages, 6 to 8 mice per cage, and were fed Derwood pellets and tap water ad libitum. Mice 20 to 25 gm. in weight and 2 to 4 months of age were employed in the various experiments.

RESULTS

Azaserine Resistance in the Plasma Cell Neoplasm 70429

Azaserine resistance has been observed to develop in the plasma cell neoplasm 70429 within the time of a single transfer generation. It has been seen to occur in two different patterns. In the first, the ascites population, following a period of growth suppression, begins to grow actively in the presence of the drug. The AZR-I line is such a line. This subline was initiated on the sixty-eighth day following tumor inoculation while the animal was still being treated with azaserine. Although AZR-I was carried two further transfers on the drug, resistance was readily demonstrable after the first transfer on azaserine.[3] AZR-I has now been transferred fifty-six generations through untreated mice since the third and last transfer in azaserine-treated mice, and azaserine resistance has persisted. The data in TABLE 1 show several tests selected from the many that illustrate this point. The persistence of sensitivity to azaserine at the seventieth ascitic transfer of the sensitive line also is recorded.

The second pattern of azaserine resistance develops in another fashion. In this second example azaserine inhibits the tumor from growing, and this effect persists for an extensive period after the cessation of azaserine treatment.

TABLE 1

RESPONSE OF VARIOUS LINES OF PLASMA CELL NEOPLASM 70429 TO AZASERINE

Line	No. mice per group	Number of serial transfer generations			Cell dose inoculated × 10^6	Day of harvest	Total ascitic tumor cell numbers, in millions	
		Total	On azaserine	Since last azaserine transfer			Control, average range	Azaserine (5 mg./kg./day), average range
Sensitive	4	32	—	—	2.65	9	660 (520–840)	8.5 (1.3–22)
	5	70	—	—	1.38	10	539 (161–787)	14.8 (4.3–35)
AZR-I	5	28	3	14	1.8	10	536 (380–740)	303 (160–460)
	5	70*	3	56	1.26	10	610 (151–862)	419 (250–495)
173 DR	4	22	1	1	3.8	12	600 (440–820)	155 (40–300)
	5	27	1	6	1.85	10	320 (120–590)	325 (150–420)
	5	31†	1	10	1.48	10	361 (280–419)	606 (260–766)

* At the time of this test, AZR-I has been carried 24 months since the last azaserine-treated transfer.

† The 173 DR has been carried 19 months since the only azaserine-treated transfer.

The findings with the 173 DR line illustrate this point. This line was developed from a mouse treated with 50 daily treatments of azaserine (5 mg./kg.). The last treatment was given on the sixtieth day following tumor inoculation. At this time the mouse showed no evidence of an actively growing neoplasm. On the one hundred and seventy-third day, 113 days after the last azaserine treatment, a recurrence of the neoplasm had developed in the cervical lymph nodes. The morphologic appearance of the cells was the same as that of the original stem line 70429. Cells from a homogenate of the cervical node were introduced intraperitoneally, and a new ascitic line was begun, which has now been carried 10 transfer generations in 19 months. This line has been consistently resistant to azaserine. Rapid serial transfer of this line has been prolonged by the chronicity of growth of infiltrations in various transfers since its isolation. On most occasions, however, ascites outgrowth has been prompt and has permitted the studies described in TABLE 1. This type of regrowth of 70429 has been called a delayed recurrence, and it represents an interesting model of tumor growth in that it mimics in many ways metastases in bone and subcutaneous tissues seen in human breast carcinoma long after mastectomy.

Five delayed-recurrence sublines of neoplasm 70429 have been studied thus far of the many available. All have been selected from azaserine- or DON-treated mice. Three have been resistant to azaserine or DON and two have been sensitive. These findings indicate that resistant populations may arise from cells that probably are not actively growing in the presence of the drug.

Development of DON Resistance in Mast Cell Neoplasm P 815

The unique functional features of the neoplastic mast cells in the mouse, as demonstrated by Sjoerdsma *et al.*[6] and Furth *et al.*,[3] include the ability to synthesize histamine, serotonin, and heparin. These functional capacities cannot be compared directly with those of nonmalignant mast cells, but it is likely that these specific functions are also properties of normal mouse mast cells. The mast cell neoplasm P 815, inoculated by the intraperitoneal route, disseminates to the loose peritoneal tissues and viscera and kills its host in much the same manner as a disseminated leukemia. No precipitating cause of death, other than that engendered by infiltration of vital organs, has been seen.

Mast cell neoplasm P 815 has been studied since the fourth transfer generation for its response to DON. This has afforded the opportunity of observing changes in the growth response of a single neoplasm to a specific inhibitory agent throughout the history of transplantation.

In TABLE 2 are found the survival time data resulting from various transfer generations, beginning with the fourth. It may be noted from this table that, as the tumor progresses through later transfer generations, the survival time is somewhat shorter. In the later transfers there has been some lessening of the response to DON. There has been at least a 100 per cent increase in the mean survival time in every case except one, which was only 74 per cent.

TABLE 3 shows the data on the development of two DON-resistant lines of P 815. The origin of the DON-R-I line began with the intraperitoneal inoculation of a homogenate of a subcutaneous growth of P 815 that had been

TABLE 2

EFFECT OF DON ON THE SURVIVAL TIME OF MICE INOCULATED WITH MAST CELL NEOPLASM P 815

Transfer generation	Cell dose × 10^6	No. daily DON treatments	Survival time, days				Average percentage increase in survival time
			Untreated		DON-treated		
			Average	Range	Average	Range	
4	Homog.	39	16.7	16–17	63.0	57–66	277
5	1.2	34	18.7	16–20	59.1	55–62	216
6	1.5	51	15.2	12–18	73.2	60–106	381
14	0.52	48	15.2	14–18	46.1	36–70	203
21	0.01	33	16.9	16–21	43.2	38–56	155
23	0.82	33	16.0	11–19	36.8	32–46	130
31	1.35	29	19.3	15–21	33.6	30–37	74
35	0.68	33	17.0	15–19	38.2	14–46	124
44	0.1	31	17.7	17–19	36.0	32–39	103
53	0.1	26	14.5	14–15	35.1	24–42	142
55	0.875	28	16.7	16–19	38.1	31–44	128
60	0.1	29	13.2	12–14	31.7	26–34	140
61	0.1	27	13.7	13–15	31.5	22–35	130
62	0.1	29	13.25	12–14	31.7	27–35	131

carried as a subcutaneous neoplasm for the first three transfer generations. The intraperitoneal introduction of cells produced ascites in both treated and untreated animals. In the fourth transfer generation the untreated mice died between the sixteenth and seventeenth days. The DON-treated mice inoculated with the homogenate following a prolonged period of growth inhibition developed ascitic tumor cell populations. One such mouse was selected at the sixtieth day. At this time, ascites and infiltration by cells of P 815 were apparent. Ascitic cells were removed and transferred. In this transfer generation (gen. 5) the untreated mice died between the twenty-first and twenty-fifth day, and the DON-treated mice between the thirty-ninth and forty-seventh day. The percentage of increase in survival time had changed from 277 (gen.-4 sensitive cells) to 95.1 per cent (gen. 5). A considerable degree of resistance had developed. After three transfer generations through DON-treated hosts (a time period of 114 days), cells then tested for their response were very resistant to DON (gen. 7). This line was transferred through a total of 6 transfer generations, through DON-treated hosts a total time period of 175 days and, thereafter, to untreated hosts. At the twelfth transfer generation DON-R-I had then completed 6 transfers in DON-treated hosts, and 2 transfers in untreated hosts. A survival time study of twelfth-transfer generation cells showed that the line was solidly resistant to DON. The next survival time studies of the DON-R-I line were done at transfer generations 22 and 36. On these occasions there was a definite loss in the degree of resistance. A comparison of survival time periods of untreated hosts with those of similar transfer generation data from the stem line cells reveals that the DON-R-I line consistently kills its host at a later time.

The development of the DON-R-II line is also recorded in TABLE 3. An

TABLE 3

DEVELOPMENT OF RESISTANCE TO DON IN MAST CELL NEOPLASM P 815

Transfer generation	Cell dose × 10^6	Age in days when transferred	No. serial transfers		Survival time, days*				Average percentage increase in survival time
			On DON	Since last DON transfer	Untreated		DON-treated		
					Average	Range	Average	Range	
DON-R-I									
4	Homog.	—	0	0	16.7	16–17	63	57–66	277
5	0.63	60	1	0	22.8	21–25	44.5	39–47	95.1
6	0.108	21	2	0	27.3	26–29	35	33–40	28.2
7	1.7	33	3	0	29.5	25–38	31.2	28–36	5.7
12	1.0	—	6	2	26.5	26–27	26.6	25–28	<1
22	1.17	—	6	12	20.7	18–23	30.5	28–32	47.3
36	0.975	—	6	26	22.5	22–23	32.5	29–36	44.4
DON-R-II									
44	0.001	—	0	0	20.5	19–25	42.8	35–49‡	108.7
45	0.001	33	1	0	20.5	20–22	21.5	20–23	4.9
46	0.0011	—	1	1	19.8	19–23†	23.2	20–25	17.2
47	0.0011	—	1	2	21.5	19–28	21.8	19–23	1.4
48	0.0013	—	1	3	20.3	18–21	24.2	21–26	19.2
50	0.001	—	1	5	19.1	18–20	23	21–25	20.2
54	0.001	—	1	9	17.1	14–18	19.8	18–22	15.8
57	0.001	—	1	12	18.0	16–20	18.2	16–20	1.1

* There were usually 8 mice in each group, and occasionally only 6.
† One survivor.
‡ Two survivors.

inoculum of 1000 sensitive cells was given to each of the mice, which were divided into 2 groups of 8 each. Untreated mice died between the nineteenth and twenty-fifth days. Of the 8 DON-treated mice, 6 died with infiltration and ascites between the thirty-fifth and forty-ninth days. Two of the mice have survived. The average percentage increase in survival time of 108 per cent pertains only to the 6 mice that died. Ascitic tumor cells were removed from one of the DON-treated animals that developed ascites and infiltration by the thirty-third day. These cells were the source of the DON R-II line, which was transferred only a single transfer generation in DON-treated mice at a total period of 33 days. The complete history of this line is given in TABLE 3. In each study, for purposes of standardization, about 10^3 cells were used as inoculating doses. It may be seen that there is little alteration in the death time of untreated mice inoculated with 1000-cell doses and in various transfer generations. The average percentage increases in survival time since removal from the DON-treated host vary from 1.1 to 20.2 per cent in the various transfer generations.

Development of Resistance to A-Methopterin in the Near-Tetraploid Lymphocytic Neoplasm P 288

The growth of lymphocytic neoplasm P 288, like many other (but not all) DBA/2 lymphocytic neoplasms is inhibited by A-Methopterin. Survival time data for mice inoculated with P 288 are given in TABLE 4. It may be

TABLE 4

EFFECT OF A-METHOPTERIN ON THE SURVIVAL TIME OF MICE INOCULATED WITH LYMPHOCYTIC NEOPLASM P 288

Transfer generation	No treatments	Day last treatment given	Cell dose	Survival time, days*				100-Day survivors	Average percentage increase in survival†
				Control		A-Methopterin			
				Average	Range	Average	Range		
4	6	13	1.74×10^6	13.5	12–16	24.1	21–28	0	78.5
19	8	15	8.5×10^5	12.1	8–18	23.8	21–30	0	96.7
49	9	20	4×10^5	10.0	7–11	26.1	24–28	0	161.0
50	11	22	1×10^4	12.5	11–14	41.3	37–52	1	231
59‡	7	15	1.03×10^7	9.0	7–11	26	21–29	0	188
59‡	7	15	2.12×10^5	10.5	10–11	29.3	24–39	0	179
59‡	7	15	2.12×10^2	15.8	12–17	34.5	27–41	2	118
122	7	18	1×10^4	11.6	11–12	24.2	19–28	0	108
122	7	18	1×10^3	12.1	12–13	34.2	28–52	1	182
122	7	18	1×10^2	12.8	11–15	38.3	32–42	5	198
140	9	25	1×10^3	13.0	9–16	33.1	29–39	0	154

* There were 8 mice in each group.

† The survivors were not used in calculating the percentage increase in survival.

‡ A-Methopterin treatment was begun the day after tumor inoculation for all groups except the G59 series. In this series, treatment was begun on the day of tumor inoculation. A-Methopterin was given 3 mg./kg. 3 times weekly on alternating days.

seen from this table that in almost all cases there was more than a 100 per cent increase in survival time. One pertinent feature of A-Methopterin, in contrast to azaserine or DON, is that toxicity to this compound limits the number of treatments that can be given. This is true when A-Methopterin is administered 3 times weekly on alternating days. When (BALB/c × DBA/2)F_1 hybrid mice are treated according to this schedule, they tolerate two weeks of treatment quite well. In the third week, however, mice develop toxicity. This varies from experiment to experiment. As may be seen in TABLE 4, from 6 to 11 treatments were given in the various experiments. The time interval between the last day A-Methopterin was given and the day the first A-Methopterin-treated mouse died varies from 1 to 19 days, with an over-all average of 9.3 days for all experiments. Sustained treatment was therefore not possible in most cases.

The data tabulated in TABLE 4 also include the effect of varying cell doses on survival time. No mouse receiving 10^5 or more cells and subsequently treated with A-Methopterin has survived indefinitely. There have been survivors, however, when 10^4 or fewer cells have been inoculated.

In attempts to develop A-Methopterin-resistant lines, multiple transfers through A-Methopterin-treated mice were required. Cell populations that develop in mice during the course of a single transfer generation have not developed full resistance to A-Methopterin. This may be due in part to the technical inability to maintain and administer A-Methopterin continuously, as was feasibly done with the glutamine antagonists, azaserine, and DON. In TABLE 5 are tabulated three characterizations of cells removed from mice

TABLE 5

RESPONSE OF P 288 CELLS ISOLATED FROM MICE FOLLOWING A SINGLE TRANSFER GENERATION ON A-METHOPTERIN

Transfer generation	Source	Cell dose inoculated	A-Methopterin dose (mg./kg.)	Total No. treatments with A-Methopterin	Day last treatment given	Days cells isolated from A-Methopterin-treated mouse	Survival time, days		Average percentage increase in survival
							Control, average range	A-Methopterin, average range	
49	Sensitive	4×10^5	3	9	20	24	10.0 (7–11)	26.1 (24–28)	161
50	A-Methopterin-treated	3.1×10^4	3	10	21		14.2 (11–17)	31.0 (24–41)	118
50	Sensitive	1×10^4	1	14	30	25	12.5 (11–14)	27.8 (19–34)	122
51	A-Methopterin-treated	3.2×10^4	1	12	25		15.8 (15–17)	29.6 (25–40)	87.5
122	Sensitive	1×10^3	3	7	18	26	12.1 (12–13)	34.2 (28–52)*	183
123	A-Methopterin-treated	1.25×10^3	3	8	20		13.3 (11–14)	25.0 (24–27)*	87.8

* Does not include 1 survivor.

treated on A-Methopterin during a single transfer generation. It may be seen that in each case the isolated cells showed some change in response to the compound, that is, some resistance. Full resistance did not develop in a single generation to the lower doses of A-Methopterin (1 mg./kg.).

An A-Methopterin-resistant P 288 subline (AMR-II) has been developed. Data on the response of this line are presented in TABLE 6. Serial study of each transfer generation was not followed for this line. However, after 19 transfer generations on A-Methopterin, a stable heritable resistant line was developed. After 14 transfers through untreated hosts, resistance has remained.

Data on the development of A-Methopterin resistance in P 288/AMR-III also are presented in TABLE 6. All untreated mice inoculated with 1000 sensitive cells died on the twelfth and thirteenth days. The 8 A-Methopterin-treated mice received a total of 7 treatments, 3 mg./kg. each. The last treatment was given on the eighteenth day. At this time there was no evidence of tumor growth. On the twenty-sixth day, ascites developed in 6 of the 8 mice. One mouse was tapped on the twenty-sixth day, and inocula of 1000 cells each were given to each of 16 mice used in the succeeding transfer. This procedure was repeated for each of the 7 transfers listed in TABLE 6. The initiating cell dose for each new transfer was approximately 10^3 cells.

The study of P 288 AMR-III has not been finished and will be complete only when resistance becomes complete. Following this, to test its heritable stability, the line will be passed serially through untreated hosts. It may be

Table 6

Development of Resistance to A-Methopterin: Lymphocytic Neoplasm P 288*

Line	Serial transfer generations		Tumor age, in days when:		Survival time, days				Average percentage increase in survival time
	On A-Methopterin	Since last A-Methopterin	Transferred	Last A-Methopterin given	Untreated		A-Methopterin-treated		
					Average	Range	Average	Range	
AMR-II†	15	0	6	—	13.6	11–14	13.6	11–14	0
	19	14	—	—	12.1	11–14	13.6	10–17	12.4
AMR-III	0	0	—	18	12.1	12–13	34.2	28–52	183
	1	0	26	20	13.3	11–14	25.0	24–27	87.8
	2	0	24	24	16.2	15–17	23.7	21–25	46.2
	3	0	19	21	17.6	16–20	23.5	22–26	33.5
	4	0	20	24	19.8	15–33	23.0	20–26	16.2
	5	0	21	17	18.8	17–21	20.6	18–25	9.5
	6	0	17	21	16.8	16–19	22.0	19–24	31.0
	7	0	—	—	18.1	16–21	20.3	17–22	12.1

* The inoculating dose in all cases was 1 × 10^3 cells. There were usually 8 mice in each group, but occasionally only 6.

† AMR-II was separated at transfer generation 105; AMR-III at generation 122.

seen from the study thus far that the cells isolated after the second transfer through treated mice now develop ascites while on treatment. The average percentage increase in survival time has been reduced during the transfer process through A-Methopterin-treated mice. The average survival time of A-Methopterin-treated mice, however, has changed from 34.2 days in the sensitive (not including the 100-day survivor), to 20 to 23 days in later transfers. This value for the average survival time of A-Methopterin-treated mice from the third to the seventh transfers has undergone very little change. However, there has been a change in the average survival time of untreated mice. With sensitive cell inocula, this value was 12.1 days, but increased from 16 to 18 days in later transfers.

Discussion

The studies reported here describe some of the techniques involved in developing resistant lines. In specific observations with plasma cell neoplasm 70429 and mast cell neoplasm P 815, 2 patterns for the development of resistant lines with each neoplasm were described. It was seen that the AZR-I subline of 70429 developed from cells that grew actively in the presence of azaserine. The 173 DR subline of 70429 was developed from cells that did not grow actively during the time at which azaserine was administered, but appeared to do so long afterward in the form of a delayed recurrence. The two DON-resistant sublines isolated from mast cell neoplasm P 815 also developed in a different manner. The DON-R-I line required multiple transfers on DON. The DON-R-I cells killed the untreated mice more slowly than the parent sensitive line. The DON resistance developed after 6 transfers through

DON-treated mice with this subline and, subsequently, underwent a reduction in degree of resistance. The DON-R-II subline of P 815, in contrast, developed during a single transfer generation within 33 days. When compared with the parent sensitive line, there was no change in the ability of these cells to kill their hosts. The DON resistance in P 815/DON-R-II has remained a stable, heritable characteristic through 12 transfer generations in untreated mice.

Two different patterns for the development of resistance to a single agent were described for plasma cell neoplasm 70429, and two for mast cell neoplasm P 815. In a careful study with a single neoplasm and a single selecting agent, the number of different patterns for the development of resistance could probably be obtained. In view of the wide variation in resistant cell types obtained in bacteria,[9] this would not be a surprising result. Careful exploration of this point, employing different quantitations in dosage of the selecting agent, is indeed warranted.

The development of resistance to A-Methopterin described in this report required multiple serial transfers of cells through A-Methopterin-treated hosts. One factor relevant to this result was that the mice developed toxicity to the compound, and treatment was discontinued. Subsequently P 288 cells grew out and killed their hosts in all cases when 10^5 or more cells were inoculated, but not in all cases when 10^4 or less cells were given. There was described a wide variation in the number of days between the time that A-Methopterin treatment was stopped and the day the first treated animal died of neoplasm. Presumably, following the course of treatment, many cells were not killed. When the effects of the A-Methopterin waned, the cells then grew. Cells that appear in mice following a single treatment schedule in the period of one transfer generation have shown some degree of resistance to the compound. Studies on the heritable stability of a low degree of resistance have not been undertaken.

The reason for the requirement of multiple transfers of cells through A-Methopterin-treated hosts is not known. A similarity to certain bacterial studies with penicillin resistance has been cited.[10] This comparison indicates that, in order to develop a stable, heritable penicillin-resistant bacterial cell type, a multiplicity of genetic changes, that is, a series of mutations, is required. From this hypothesis, then, it is unlikely that a completely stable, heritable resistant cell type could antedate experience with the selecting agent. Recently, Skipper *et al.*[11] have reported studies with two lymphocytic neoplasms of mice, L 1210 and L 4946. These authors speculated that the inability to cure mouse leukemia was related to the inoculating dose of cells. For example, with lymphocytic neoplasm L 4946, when 10^4 or less cells were inoculated, total eradication of the leukemic cells was observed in some mice treated with A-Methopterin. Skipper *et al.* speculated that resistant cells were present in sufficient concentration in 10^5 and larger doses of cells to account for the leukemic deaths.[11] This speculation may be proved only by characterization of the cells that grew out and killed A-Methopterin-treated hosts. Were these cells resistant? It would be surprising if they were; indeed, as Skipper cites in some cases, such cells have not been found to be resistant.[12] The principal reasons for this speculation are as follows. First, it has not been demonstrated that A-Methopterin or other antimetabolites destroy all sensitive cells, leaving behind resistant forms. Second, the role of physiological adaptation to A-Methopterin has been neglected as a consideration. In general, tumor cells may possess abilities to adapt physiologically, rather than genetically, to antimetabolite inhibition.

One basic question apparent throughout the studies reported here is concerned with the index one should employ to measure the degree of resistance. In the tables, the average percentage increase in survival time was used. However, this index can be quite misleading when changes occur in the killing time in control mice. A biomathematical and statistical appraisal of this index is needed. In this respect it is important to note that in two lines, P 815/DON-R-I and P 288/AMR-III, there was a prolongation in the killing time by cells that had been transferred through A-Methopterin-treated hosts.

It would seem that the average percentage increase in survival time is not the best index of degree of resistance. Future statistical approach to this question must deal with the problem of survivors. What value do 100-day survivors assume in the average survival time calculation? Future work on the development of resistant lines also should consider certain quantitative problems relevant to the dosage level of the selecting agent. There may be many stable, heritable resistant forms emerging in any population exposed to the selective pressure of an antimetabolite that can resist low, but not high, levels of the antimetabolite. In any chemotherapeutic study it will be important to determine whether cells possessing a low level of resistance can grow out and kill their hosts. In all cases employed here, relatively high doses of the selecting agents were employed. The above questions and remarks are directed at a biological system in which genetic adaptation is the principal mechanism for resistance. If physiological adaptation enters into the biological process of resistance, however, many further difficulties will be encountered. A facile system of cloning may be one of the ways in which these problems can be resolved.

Much of the work on drug resistance in tumor cell systems has been interpreted in light of the well-controlled studies of resistance to antibiotics in bacteria. There are certain dangers inherent in these comparisons. The somatic tumor cell system is not subject to controlled genetic study. Further, there may be other biological mechanisms whereby somatic cells may develop new characteristics that are heritable in nature. The phenomenon of differentiation and development probably permits cells of a common genotype in a highly integrated organism to develop the many histological and biochemical characteristics of differentiated cells. Foulds[13] has pointed out the relevance of epigenesis to the cancer problem. Karyologic studies with tumor cells have indicated that many transplanted tumors are subject to disturbances in chromosome reproduction. This general phenomenon may result in alterations in chromosome number, type, and size.[14, 15] A process of this nature could well upset the whole organization of the cell and, specifically, any unit character, including drug resistance, under consideration.

In future work with somatic cell systems it will be important to separate carefully the heritable and genic entities. The fact that a property of a somatic cell is stable and heritable does not imply that the means of transmission of that property is exclusively controlled by genes.

SUMMARY

The development and heritable stability of azaserine resistance in plasma cell neoplasm 70429 were described. The development of resistance to azaserine in this neoplasm appeared in two different patterns. In one case the neoplasm developed the ability to grow rapidly in the presence of treatment (AZR-I); in the second case the neoplastic cells were inhibited by the compound and, following a long quiescent period during which no azaserine treatment was administered, cells began to grow in the form of a delayed recurrence (173 DR).

The development of two DON-resistant sublines of mast cell neoplasm P 815 was described. The DON-R-I line required multiple transfers through DON-treated hosts and subsequently lost resistance. The DON-R-II line developed resistance to DON in the course of 33 days in a single transfer generation and has remained resistant through 12 transfers through untreated mice.

The development of A-Methopterin resistance in a near tetraploid of the lymphocytic neoplasm P 288 was described.

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TRANSPLANTABLE LYMPHOMAS IN RATS AND RESPONSIVENESS TO DRUGS*

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This report will be confined to a discussion of the growth characteristics and responsiveness to a few standard drugs of three transplantable rat neoplasms.

The first, Yoshida sarcoma, was described by Yoshida[1] to be transplantable as an infiltrative lymphoma or an ascites sarcoma. The tumor was observed in 1943 in one of 20 albino rats of mixed breed that had been fed *o*-amidoazotoluol and painted with potassium arsenite. The primary tumor, a round-cell or reticulum-cell sarcoma was observed in the scrotum of the treated rat and was found to invade the pelvic cavity and the retroperitoneal tissues. It proved to be transplantable in 90 per cent of the rats of the same mixed breeds.

We obtained the transplanted sarcoma and breeding stock from the Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y., in May 1953, where both had been received from Yoshida some time previously. From the foundation stock the descendants of a single pregnant female have been bred brother by sister in our laboratory for nine generations, and the sarcoma has been transplanted for the hundredth transfer generation. When transplanted subcutaneously as a 2- to 3-mg. graft of tissue, the sarcoma becomes palpable and can be measured by the tenth to thirteenth day after inoculation. FIGURE 1a shows a representative section through the subcutaneous growth 14 days after inoculation. Death of the host usually occurs from the fourteenth to the twentieth day after inoculation. The growth infiltrates the skin and underlying muscle, and metastases to the lung (FIGURE 1b), mesentery, colon (FIGURE 1c), and heart (FIGURE 1d) are common. It grows progressively in nearly 100 per cent of the inoculated rats.

The second neoplasm, R2788, is a spontaneous lymphosarcoma of the illeocolic mesentery like those described by Curtis and Dunning[2] in 1940. The primary tumor was observed in 1947 in a piebald A × C line 9935 rat of the thirtieth brother-by-sister generation of the line. It is currently in the one hundred and thirty-eighth transfer generation and grows progressively in 100 per cent of the inoculated rats of this line.

Histologically, R2788 is composed of relatively large lymphoblasts with very little cohesion or supporting structure. Rats inoculated bilaterally with 2- to 3-mg. grafts of tissue usually succumb by the twenty-fifth to thirty-fifth day after inoculation, with large subcutaneous masses (FIGURE 2a) and extensive involvement of the superficial (FIGURE 2b), mediastinal, mesenteric, and retroperitoneal (FIGURE 2c) lymph nodes. The subcutaneous growth can be measured 17 to 20 days after inoculation.

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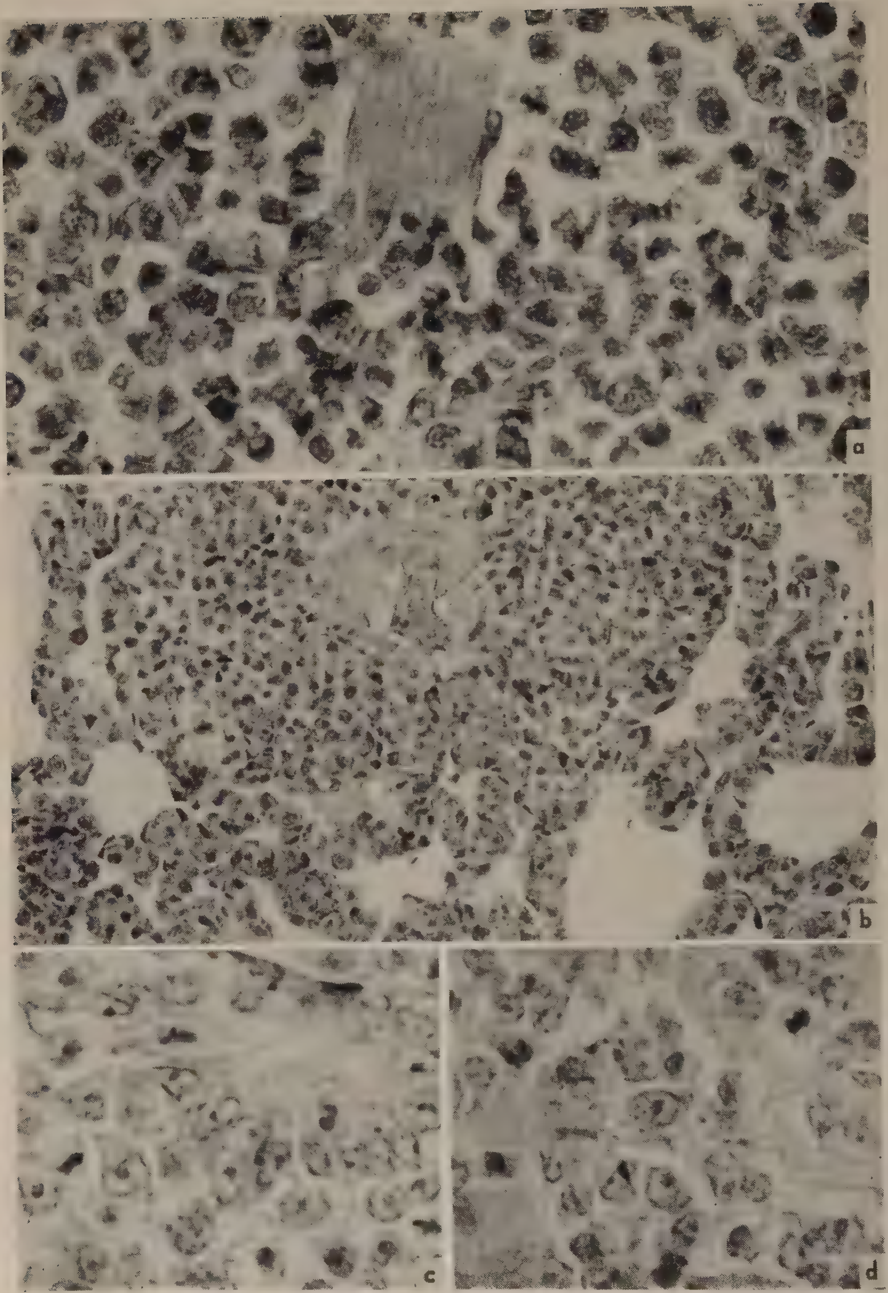

FIGURE 1. (a) Section from a subcutaneous tumor 14 days after inoculation of YOSA/23A (×353); (b) section through a metastasis in the lung 14 days after inoculation with YOSA/23A (×156); (c) section from a metastasis in the wall of the colon 14 days after inoculation of YOSA/23A (×353); (d) section from a metastasis in the heart 14 days after inoculation with YOSA/23A (×353).

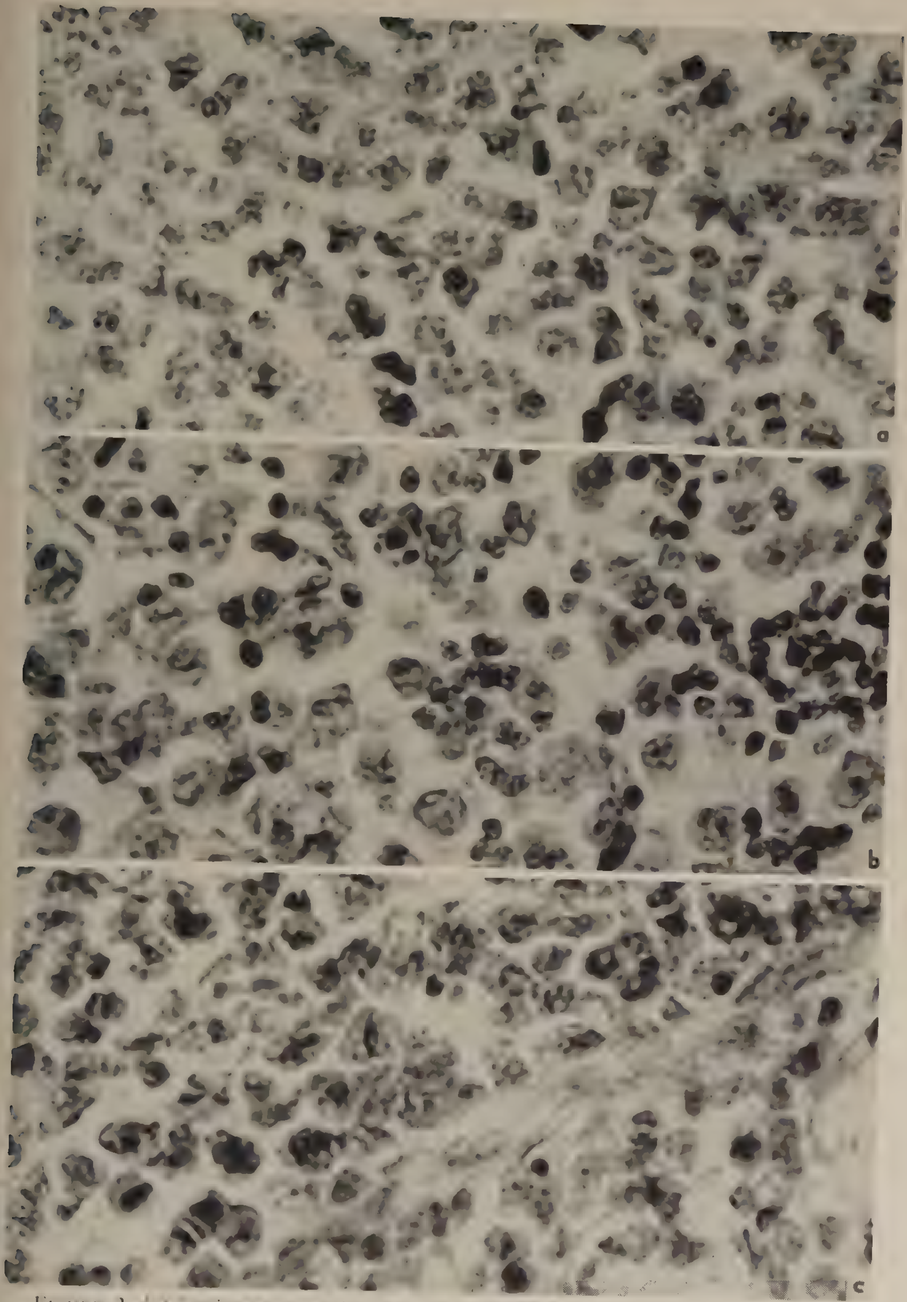

FIGURE 2. (a) Section from a subcutaneous tumor 29 days after inoculation with R2788/78A (×353); (b) section from an axillary lymph node metastasis, showing the relative size of normal lymphocytes and malignant cells 29 days after inoculation with R2788/87A (×353); (c) section from a retroperitoneal lymph node metastasis involving a capsule of adrenal 29 days after inoculation with R2788/79A (×353).

The third neoplasm, IRC741, is an acute leukemia,[3] probably of the monocytic type. It occurred spontaneously in a Fischer line 344 rat of the fifty-first brother-by-sister generation of the line and is currently in the one hundred and thirty-fifth transfer generation. It is transplantable by subcutaneous trocar implantation of 2- to 3-mg. grafts of tissue, or by subcutaneous or intraperitoneal injection of liver or spleen suspensions or whole or diluted peripheral blood. It grows progressively in 100 per cent of the inoculated rats of the line of origin.

For the current studies, IRC741 has been transplanted subcutaneously by trocar on the right side as 2- to 3-mg. grafts of tissue taken from the site of a previous inoculation. The subcutaneous growth is palpable 10 days after inoculation. The peripheral blood usually contains a high percentage of malignant cells in a count of 30,000 or above from the thirteenth day after inoculation. Death usually occurs between the fourteenth and twentieth days

TABLE 1

RESPONSE OF EACH NEOPLASM TO A MINIMAL EFFECTIVE DOSE OF NITROGEN MUSTARD

Tumor/ generation	Total dose		Number of injections	Number:		Mean days:		Mean tumor diameter:		Percentage increase in survival	Percentage decrease in tumor diameter
	mg./kg.	mg.		rats	tumors	to death ± P.E.	diff. ± P.E.	in cm. ± P.E.	diff. ± P.E.		
								13 Days			
YOSA/74A	Control			9	9	21 ±1.3	8 ±1.6	3.7 ±0.09	1.9 ±0.18	38	51
	1.1	0.2	4	9	7	29 ±0.89		1.8 ±0.16			
								17 days			
R2788/118A	Control			10	20	28 ±0.33	3 ±0.61	3.4 ±0.07	1.3 ±0.14	11	38
	1.6	0.4	4	10 (IT)	18	31 ±0.51		2.1 ±0.12			
								15 Days			
								WBC			
IRC741/120A	Control			10	10	19 ±0.50	13 ±0.66	79,300		68	
	1.0	0.12	5	10	10	32 ±0.43		20,300			

after inoculation; post-mortem examination reveals an edematous, hemorrhagic growth infiltrating the skin and muscle at the site of inoculation, large hemorrhagic adjacent axillary and mediastinal lymph nodes, and generalized involvement of the lungs, liver, and spleen.

Tables 1 to 6 show the response obtained for each of the three neoplasms to a comparative dose of each of the 6 following compounds: nitrogen mustard; Nitromin; Myleran; 6-mercaptopurine; Thio-TEPA; and Methotrexate. Each of the compounds, in aqueous or saline solution, was injected intraperitoneally every other day or every fourth day, beginning 24 hours after inoculation of the tumor.

The rats were weighed before inoculation and weekly thereafter. The tumors were measured as soon as practicable after they were palpable, and weekly thereafter. The three largest diameters were recorded in centimeters for each tumor, and an average diameter was obtained. After completion of the injection schedule the rats were observed until death for further growth of tumors, average survival time, and post-mortem examination. A representative section of each tumor, the axillary and mediastinal lymph nodes, four sections of lungs, and a section of liver, spleen, and kidney were preserved for microscopic examination.

Table 1 shows the response of each of these neoplasms to a minimal effective dose of nitrogen mustard. Rats inoculated with YOSA/74A received 4 injections of 0.05 mg. of nitrogen mustard beginning the day after inoculation and on every fourth to sixth day thereafter, or a total dose of 0.2 mg. or 1.1 mg./kg. The untreated controls died with tumors in 21 ± 1.3 days, while

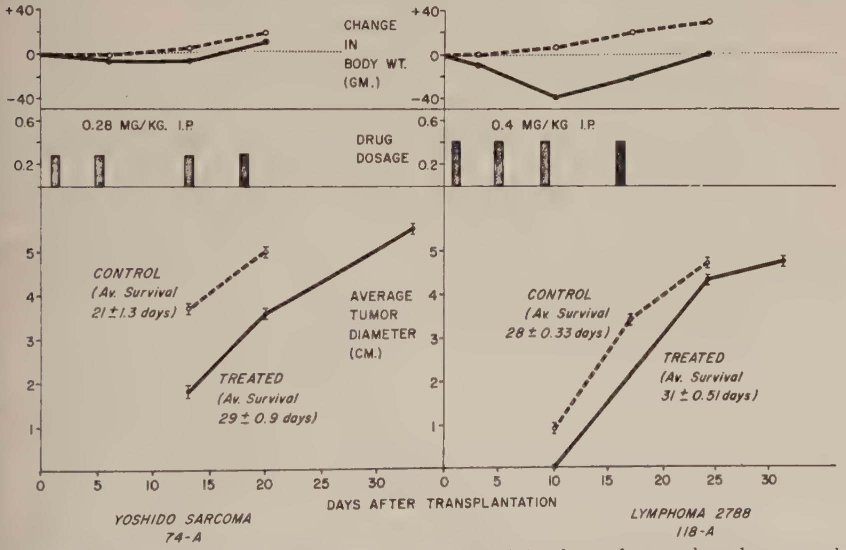

Figure 3. Dosage schedule of nitrogen mustard in inbred rats for two lymphomas and a comparison of the average change in body weight and tumor diameter for the treated and control groups.

only 7 of the 9 treated rats developed tumors, and these survived an average of 8 ± 1.6 days, or 38 per cent longer. The tumors were measured on the thirteenth day after inoculation. In the treated rats the diameters averaged 1.8 ± 0.16 cm., compared with 3.7 ± 0.09 cm. for the controls; that is, the treatment reduced the average size of the tumors by 51 per cent.

From the same table it may be seen that rats inoculated with R2788/118A

TABLE 2

RESPONSE OF EACH NEOPLASM TO NITROMIN ADMINISTERED EVERY FOURTH DAY

Tumor/generation	Total dose		Number of injections	Number:		Mean days:		Mean tumor diameter:		Percentage increase in survival	Percentage decrease in tumor diameter
	mg./kg	mg.		rats	tumors	to death ± P.E.	diff. ± P.E.	in cm. ± P.E.	diff. ± P.E.		
								13 Days			
YOSA/83A	Control			8	8	18 ±0.63	11 ±0.80	4.1 ±0.05	2.5 ±0.25	61	61
	3.4	0.8	4	8	5	29 ±0.49		1.6 ±0.25			
								20 Days			
R2788/132A	Control			9	18	26 ±0.83	9 ±1.5	3.9 ±0.07	1.8 ±0.16	35	46
	23.4	6.0	6	9	18	35 ±1.24		2.1 ±0.14			
								13 Days			
								WBC			
IRC741/135C	Control			12	12	15 ±0.24	9 ±0.35	126,300		60	
	5.0	0.3	4	5	5	24 ±0.26		23,000			
								14 Days			
								WBC			
IRC741/132C	Control			9	9	15 ±0.02	17 ±0.81	169,800		115	
	16.0	0.9	4	9	4	32 ±0.81		15,300			

received 4 injections of 0.1 mg. of nitrogen mustard on the same schedule. They received a total dose of 1.6 mg./kg., which increased the average survival time by only 11 per cent, and reduced the average diameters of the tumors measured on the seventeenth day after inoculation by 38 per cent. FIGURE 3 shows graphically the details of this experiment. It is obvious that the Yoshida sarcoma showed a greater response to a nontoxic dose of nitrogen mustard than did R2788 to a dose that caused considerable loss in body weight.

The rats inoculated with IRC741/120A received 5 intraperitoneal injections of 0.024 mg., or a total of 1.0 mg. kg., and this treatment increased the average survival time of the treated rats by 13 ± 0.66 days (68 per cent). The WBC of the peripheral blood taken on the fifteenth day after inoculation showed an average of 79,300 white cells/cu. mm. for the untreated controls and 20,300 for the rats treated with nitrogen mustard.

These data seem to indicate that R2788 is somewhat less responsive to the nitrogen mustard treatment than the Yoshida sarcoma or the acute leukemia IRC741.

Similar data are given in TABLE 2 for treatment with Nitromin every fourth day. These data show that a dose of 3.4 mg./kg. reduced the number of tumors by 38 per cent in the rats inoculated with YOSA/83A. The average diameter of the tumors at 13 days was reduced by 61 per cent, and the average survival period of the treated tumor bearers was increased by 11 ± 0.80 days (61 per cent).

Seven times the above dose, or 23.4 mg./kg., failed to prevent the growth of any of the isologously transplanted R2788 lymphosarcomas. It increased

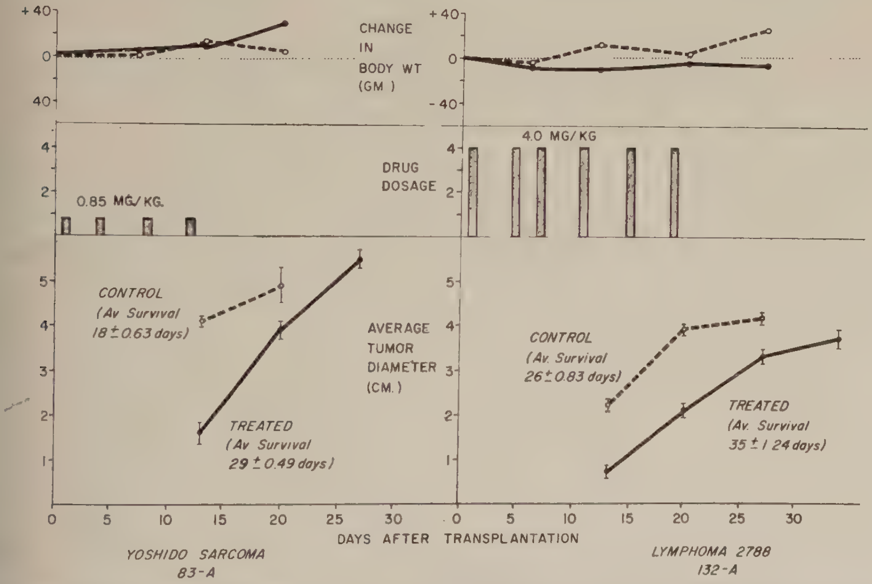

FIGURE 4. Dosage schedule of Nitromin in inbred rats for two lymphomas and a comparison of the average change in body weight and tumor diameter for the treated and control group.

the average survival period of the tumor bearers by 9 ± 1.5 days (35 per cent). The details of this experiment are shown graphically in FIGURE 4.

For IRC741, a dose of 5.0 mg./kg. increased the survival period of the leukemic rats by 9 ± 0.35 days (60 per cent). A dose of 16.0 mg./kg. reduced the number of leukemic rats by more than 50 per cent and increased the average survival period of the rats that developed leukemia by 17 ± 0.81 days (115 per cent). The WBC/cu. mm. of the peripheral blood of the untreated control rats averaged 169,000 on the fifteenth day after inoculation, compared with 15,300 for the rats treated with Nitromin.

All three of the neoplasms responded to nontoxic doses of Nitromin, but R2788 required a larger dose than either of the other neoplasms.

TABLE 3 shows similar data for rats treated with Myleran every other day. A total dose of 17 mg./kg. proved to be 50 per cent toxic to Yoshida rats.

TABLE 3

RESPONSE OF EACH NEOPLASM TO MYLERAN ADMINISTERED EVERY OTHER DAY

Tumor/generation	Total dose		Number of injections	Number·		Mean days:		Mean tumor diameter·		Percentage increase in survival	Percentage decrease in tumor diameter
	mg./kg	mg.		rats	tumors	to death ± P.E.	diff. ± P.E.	in cm. ± P.E.	diff. ± P.E.		
								13 Days			
YOSA/79A	Control			7	7	22 ±1.0	4.0 ±1.0	2.7 ±0.31	1.8 ±0.34	18	67
	17	4.0	4	6	3*	26 ±0.50		0.9 ±0.15			
								19 Days			
R2788/127A	Control			9	18	28 ±0.74	7 ±1.6	3.7 ±0.06	0.6 ±0.09	25	16
	19	5.0	5	9	18	35 ±1.4		3.1 ±0.07			
								15 Days			
								WBC			
IRC741/119A	Control			10	10	20 ±0.14	2 ±0.52	43,400		10	
	27	2.7	9	10	10	22 ±0.50		8,900			

* Three toxic deaths in 16 to 20 days with no tumor.

It increased the average survival period of the surviving tumor bearers by only 18 per cent. A similar dose of 19 mg./kg. was not toxic to A × C rats inoculated with R2788, but it increased their average survival period by only 7 ± 1.6 days (25 per cent). A somewhat larger dose of 27 mg./kg. increased by only 2 ± 0.52 days (10 per cent) the average survival time of Fischer line 344 rats inoculated with IRC741. However, the average WBC/cu.mm. for the rats treated with Myleran was 8900 on the fifteenth day after inoculation, compared with an average of 43,400 for the untreated control rats.

Similar data are shown in TABLE 4 for rats treated with 6-mercaptopurine. A total dose of 61 mg./kg. of 6-mercaptopurine injected every other day increased by 14 ± 3.1 days (78 per cent) the average survival period of rats inoculated with the Yoshida sarcoma. A slightly higher dose of 88 mg./kg. in 8 injections increased by only 26 per cent the average survival period for rats bearing lymphosarcoma R2788. An increase of 15 ± 0.78 days (79 per

Table 4

Response of Each Neoplasm to 6-Mercaptopurine

Tumor/generation	Total dose		Number of injections	Number:		Mean days:		Mean tumor diameter:		Percentage increase in survival	Percentage decrease in tumor diameter
	mg./kg	mg.		rats	tumors	to death ± P.E.	diff. ± P.E.	in cm. ± P.E.	diff. ± P.E.		
								13 Days			
YOSA/67A	Control			8	8	18 ±1.2	14 ±3.1	3.6 ±0.12	1.6 ±0.30	78	44
	61	13	7	6	6	32 ±2.9		2.0 ±0.28			
								20 Days			
R2788/120A	Control			9	18	31 ±1.0	8 ±1.1	3.4 ±0.10	2.0 ±0.11	26	59
	88	20	8	10	18	39 ±0.55		1.4 ±0.06			
								14 Days			
								WBC			
IRC741/115A	Control			10	10	19 ±0.14	15 ±0.78	51,600		79	
	156	24	12	10	10	34 ±0.77		15,700			

cent) in the average survival period of rats inoculated with IRC741 resulted from treatment at twice the above level, or 156 mg./kg. This was nearly three times the dose required to evoke a similar response in the rats inoculated with the Yoshida sarcoma.

Comparative results with Thio-TEPA are shown in TABLE 5. A total dose of 1.6 mg./kg. injected in 6 doses of 0.05 mg. every other day reduced by 1.6 ± 0.23 cm. (48 per cent) the average diameter of the Yoshida sarcomas on the thirteenth day. No tumors developed in rats similarly treated with a total of 14 mg./kg. Approximately 3 times this dose, or a total of 40 mg./kg., cured 30 per cent of the rats inoculated with R2788 and increased by 60 ± 3.9 days (182 per cent) the average survival period of the rats that developed

TABLE 5

RESPONSE OF EACH NEOPLASM TO THIO-TEPA

Tumor/general	Total dose		Number of injections	Number:		Mean days:		Mean tumor diameter:		Percentage increase in survival	Percentage decrease in tumor diameter
	mg./kg.	mg.		rats	tumors	to death ± P.E.	diff. ± P.E.	in cm ± P.E.	diff. ± P.E.		
								13 Days			
YOSA/99A	Control			5	5	16 ±1.5	9 ±1.8	3.3 ±0.08	1.6 ±0.23	56	48
	1.6	0.30	6	5	5	25 ±1.4		1.7 ±0.22			
YOSA/78A	Control			5	5	20 ±2.2		3.5 ±0.11			
	14	3.6	6	8	0	33+		0	100 % No tumors		
								28 Days			
R2788/111A	Control			9	18	33 ±1.5	60 ±3.9	4.4 ±0.08	4.1 ±0.13	182	93
	40	8.0	16	10	13	93 ±3.6		0.3 ±0.10 30 % No tumor			
								15 Days			
								WBC			
IRC741/118A	Control			10	10	19 ±0.14	13 ±0.64	52,900		68	
	3.8	0.60	9	10	10	32 ±0.62		10,000			
	15	2.4	9	10	0			9,000	100 % No tumors		

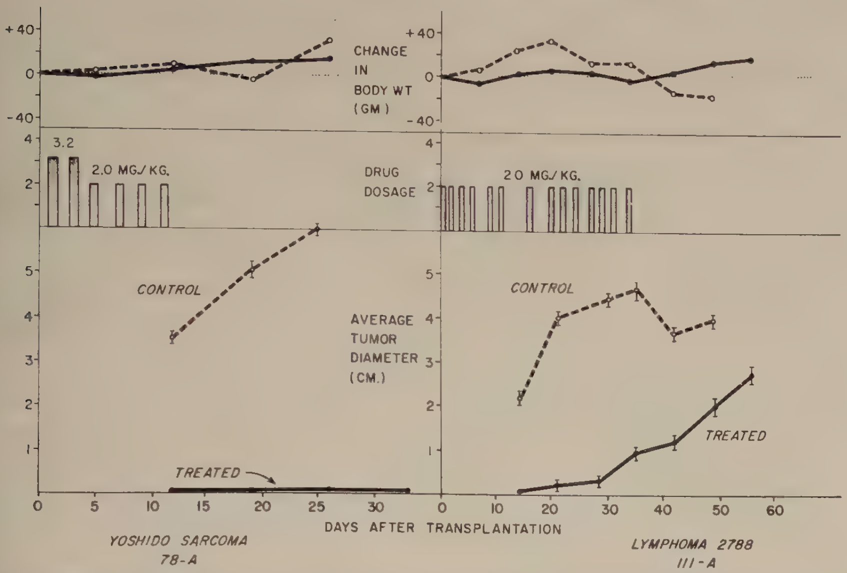

FIGURE 5. Dosage schedule of Thio-TEPA in inbred rats for two lymphomas and a comparison of the average change in body weight and tumor diameter for the treated and control groups.

tumors. The details of this experiment are shown graphically in FIGURE 5. A dose of 3.8 mg./kg. increased by 13 ± 0.64 days (68 per cent) the average survival period of rats with IRC741, while 100 per cent cures were obtained by treatment with a dose of 15 mg./kg. in 9 injections; that is, the Yoshida sarcoma and the transplanted leukemia showed about equal response to Thio-TEPA, and R2788 appeared to be somewhat more resistant.

Finally, TABLE 6 shows the response of the 3 neoplasms to Methotrexate. For rats with the Yoshida sarcoma, a minimal dose of 0.05 mg. for 3 injections every other day, or a total dose of 0.9 mg./kg., increased the average survival period and decreased by 21 per cent the average diameters of the tumors at 10 days. In the treated group there was 1 death that was probably due to toxicity. Four similar injections proved to be nontoxic to rats inoculated with R2788, and reduced the average diameters of the tumors in the treated rats by 2.4 ± 0.14 cm. (63 per cent). Twice the above dose, or 2.2 mg./kg., proved very toxic to the A × C rats. Seven of the 9 injected rats died without tumors, and the tumors in the surviving rats were reduced by 4.0 ± 0.21 cm. (71 per cent) in average diameter. These rats survived an average of 20 ± 1.7 days (77 per cent) longer than the untreated controls.

In rats inoculated with IRC741, a total dose of 9.1 mg./kg., or 4 times the above dose, proved toxic to only 2 of 10 injected rats, and increased by 14 ± 0.70 days (87 per cent) the average survival period of the rats that developed leukemia. A slightly smaller dose (not shown in the table) of 8.5 mg./kg. in 8 injections proved to be nontoxic and to increase by 9 ± 0.84 days (53 per cent) the average survival period of the leukemic rats.

TABLE 6

RESPONSE OF EACH NEOPLASM TO METHOTREXATE

Tumor/generation	Dose		Number of injections	Number:		Mean days:		Mean tumor diameter		Percentage increase in survival	Percentage decrease in tumor diameter
	mg./kg.	mg.		rats	tumors	to death ± P.E.	diff. ± P.E.	in cm. ± P.E.	diff. ± P.E.		
								10 Days			
YOSA/98A	Control			10	10	14 ±0.45	3 ±1.4	2.8 ±0.07	0.6 ±.15	21	21
	0.9	0.15	3	9	8	17 ±1.3		2.2 ±0.14			
								20 Days			
R2788/138A	Control			8	16	25 ±0.48	11 ±.96	3.8 ±0.07	2.4 ±.14	44	63
	1.2	0.20	4	8	16	36 ±0.83		1.4 ±0.13			
								23 Days			
R2788/135A	Control			10	20	26 ±0.28	20 ±1.7	4.2 ±0.07	4.0 ±.21	77	95
	2.2	0.40	4	9	4	46 ±1.7		0.2 ±0.20			
								13 Days			
IRC741/130E	Control			10	10	15 ±0.21	14 ±.70	4.0 ±0.07	WBC 119,850	87	100
	9.1	0.70	5	10	8	29 ±0.67		0	39,613		

Summary

Each of the three neoplasms showed a quantitative response to relatively nontoxic doses of the drugs tested. The Yoshida sarcoma responded to minimal doses. R2788 was less responsive than IRC741 to nitrogen mustard, Nitromin, and Thio-TEPA, and was more responsive to Methotrexate, 6-mercaptopurine, and Myleran. The homologously transplanted Yoshida sarcoma was the most responsive, possibly because of difference in the host-tumor relationship. The ultimate decision as to the relative merits of the respective neoplasms as screening tools would seem to depend upon correspondence with the response obtained in clinical experience with the tested drugs.

References

1. Yoshida, T. 1952. Studies on an ascites (reticulo-endothelial cell) sarcoma of the rat. J. Natl. Cancer Inst. **12:** 947–970.
2. Curtis, M. R. & W. F. Dunning. 1940. Transplantable lymphosarcomata of the mesenteric lymph nodes of rats. Am. J. Cancer. **40:** 299–309.
3. Dunning, W. F. & M. R. Curtis. 1957. A transplantable acute leukemia in an inbred line of rats. J. Natl. Cancer Inst. **19:** 845–853.

Discussion of the Paper

C. Chester Stock (*Sloan-Kettering Institute for Cancer Research, New York, N. Y.*): I noticed that you used different dose levels, and I know that some of them, presented as total doses, are based on differences in numbers of injections. There seem to be other differences, however, and I wonder if these represent differences in the host-tumor tolerance of the compounds.

Dunning: The dosage given in the table is the total dose in milligrams per kilogram that the rat receives, and the individual injection dose is the number of injections divided into the total dose per milligram per kilogram. There were only one or two instances where the dose was different from the injec-

Table 1
Response of WR-III to Chemotherapeutic Agents

No. of exp.	Agent	Dose* (mg./kg.)	Route	Survival			
				Mean (days)		Difference (per cent)	Evaluation
				Treated	Control		
55 TR	Methotrexate	1.5	I.P.	19.7	15	+31	±
56 TR	Methotrexate	3.0	I.P.	17.3	12	+44	±
56 TR	6-MP	75	I.P. followed by oral	40	15	+167	+++
65 TR	6-MP	75	I.P. followed by oral	19.1	12.8	+49	±
68 TR	6-MP	37.5	I.P. followed by oral	28.5	19.7	+45	±
		75	I.P. followed by oral	37.5	19.7	+90	+
		150	I.P. followed by oral	36.0	19.7	+83	+
		300	I.P. followed by oral	18.5	19.7	−6	−
59 TR	TEM	0.2	I.P.	28.3	17.0	+66	+
75 TR†	HN2	0.2	I.P.	42+	12	+250+	+++
		0.4	I.P.	94+	12	+682+	+++
61 TR	Myleran	2	I.P.	14.7	12.3	+23	±

* Dosage schedule varied with each agent employed.

† One fifth of the rats at the lower dose and four fifths of the rats at the higher dose were sacrified at 110 days.

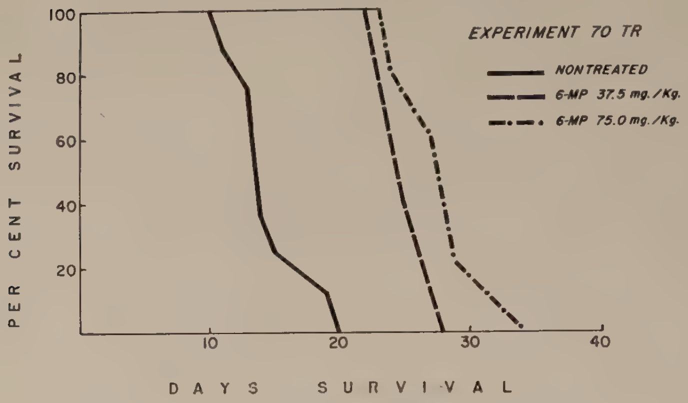

FIGURE 1. Effect of 6-MP on WR-III leukemia in WR rats.

TABLE 2
BLOOD PICTURE IN WR-III LEUKEMIA TREATED WITH 6-MP

No. of rat	Total WBC (cu. mm.)	Differential count			
		Blasts (per cent)	Lymphs (per cent)	PMN's (per cent)	NRBC (in 100 cells)
Controls: day 13					
2	89,460	28	29	29	29
3	87,860	28	28	28	28
4	114,840	38	29	17	13
6	99,840	30	30	29	22
7	93,980	36	17	36	26
Average.	97,190	32	27	28	24
Treated, 37.5 mg./kg.: day 13					
1	21,000	4	59	15	0
2	19,200	1	63	18	0
3	23,560	2	55	21	1
4	25,380	2	51	23	2
5	24,380	6	40	22	4
Average.	22,700	3	54	20	2
Treated, 75 mg./kg.: day 13					
6	21,380	4	57	24	1
7	20,000	5	51	20	0
8	15,900	3	53	26	0
9	26,580	1	58	34	12
10	12,000	0	44	30	0
Average.	19,170	3	53	27	7

TABLE 3

BLOOD PICTURE IN WR-III LEUKEMIA TREATED WITH NITROGEN MUSTARD

No. of rat	Total WBC (cu. mm.)	Differential count			
		Blasts (per cent)	Lymphs (per cent)	PMN's (per cent)	NRBC (in 100 cells)
	Controls: day 14				
1	Died on day 11				
2	Died on day 11				
3	Died on day 11				
4	63,000	25	43	20	5
	Treated 0.20 mg./kg.: day 14				
1	10,000	3	58	30	4
2	12,200	2	57	28	16
3	11,800	3	50	38	8
4	18,400	0	78	14	1
5	17,100	4	71	14	13
Average..........	14,040	2.4	62.8	24.8	8.4
	Treated 0.40 mg./kg.: day 14				
11	19,000	3	68	17	0
12	11,600	2	71	19	6
13	13,600	1	80	14	9
14	18,400	1	68	18	19
15	12,400	5	59	22	4
Average..........	15,000	2.4	69.2	18	7.6

tion. I think the Yoshida sarcoma had two doses that were larger than the three following doses, but I think this is the only instance.

Regarding Furth's comment about the resistance, in the nearly 5 years we have transplanted the IRC leukemia we have not encountered a resistant animal; and in all the cures obtained with drugs, the animals, after 90 days, have been reinoculated and have died from the disease within the expected time.

CHARLOTTE L. MADDOCK (*Children's Cancer Research Foundation, Boston, Mass.*): My associates and I have been studying the response to chemotherapeutic agents of an acute leukemia recently isolated in an inbred strain of Wistar rats. This leukemia, developed in Furth's laboratories, originated in an aging WR rat that had a mammary tumor. From many points of view it has proved to be a valuable tool in that it is species specific, 100 per cent transplantable, is not limited in its takes by age or sex and has a short survival time—usually between 12 to 16 days. Also, unlike Dunning's IRC741 leukemi ain Fischer rats, it is relatively resistant to such chemotherapeutic agents as we have employed. To date we have established no cures. In this respect it more closely resembles the situation in human leukemia.

Table 1 shows the response of this leukemia to a series of the better-known tumor-inhibitory agents. As may be seen, a significant response was obtained in one experiment with 6-mercaptopurine (6-MP). In another experiment, not yet terminated, nitrogen mustard appears to be holding the leukemia in check. Figure 1 shows graphically the increase in survival time produced by 6-MP, given at two dose levels.

The blood picture with this leukemia is sufficiently puzzling to justify designating it as a stem cell leukemia. Neither the total white count nor the percentage of blasts climb to extremely high values. Thus, total white blood cell counts remain in the neighborhood of 100,000 when in terminal stages, and percentage of blasts usually remains under 50. Nucleated red blood cells may represent 20 to 25 per cent of all nucleated cells.

Table 2 gives the blood picture in a group of control and 6-MP-treated rats on comparable days. The treated rats show an essentially normal total and differential count as compared to the nontreated. Table 3 shows the results with nitrogen mustard.

Intensive infiltration of viscera, especially liver, spleen, and lymph nodes, occurs in WR-III. A characteristic feature is the terminal hemorrhage. This may be massive or may be mere oozing, usually into the gastrointestinal tract.

Platelets may be reduced from 1,000,000/cu. mm. to 200,000. Studies of prothrombin times, sedimentation rates, and red cell fragility give essentially normal values. Further work on the causation of bleeding in this leukemia undoubtedly is indicated.

USEFULNESS OF DUNNING LEUKEMIA IRC741 FOR QUANTITATIVE PHARMACOLOGICAL STUDIES OF CANCER CHEMOTHERAPEUTIC AGENTS*

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Introduction

Leukemia IRC741 in Fischer line 344 rats, described by Dunning and Curtis in 1957[1] and in the preceding paper in this volume, has proved a useful tumor for quantitative pharmacological comparison of cancer chemotherapeutic agents. This leukemia is sensitive to alkylating agents of the bis-β-chloroethylamine and ethylenimine types, to folic acid antagonists related to Aminopterin,† and to purine antagonists such as 6-mercaptopurine; all of these materials are known to be effective in man. This leukemia is readily transplanted with 100 per cent success, and spontaneous regression has never been observed. Remarkable uniformity in the natural history of this tumor permits increase in survival time, change in white blood cell count, morphology of cells in peripheral blood and bone marrow, size of the subcutaneous tumor at the inoculation site, and extent of organ infiltration to serve as critical parameters for evaluating the effects of candidate compounds on the disease.

It is the purpose of this communication to describe the use of this tumor for quantitative pharmacological studies of potential cancer chemotherapeutic agents.

Materials and Methods

The tumor was transplanted by trocar inoculation of 2- to 3-cu.mm. fragments into the subcutaneous tissue of the right flank of Fischer line 344 rats weighing between 60 and 100 gm. (4 to 6 weeks of age). The materials to be tested were dissolved in 0.9 per cent saline or suspended in 0.5 per cent carboxymethyl cellulose in a concentration such that the volume of fluid injected with the daily dose did not exceed 1.0 ml. The materials tested were administered by intraperitoneal injection beginning 24 hours after tumor transplantation; control groups received only the appropriate vehicle. The animals were weighed before inoculation and once weekly thereafter. On the thirteenth day after inoculation a total white blood cell count and differential count were performed. When the total white blood cell count on day thirteen exceeded 30,000/cu.mm. the animal was considered to be leukemic. In about 10 per cent of the animals the total white blood cell count was unobtainable or unreliable, and these animals were considered to be leukemic if the peripheral blood smear contained immature cells. At the time of death the subcutaneous tumor was measured in three diameters, post-mortem ex-

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* The work reported in this paper was supported by Grants-in-Aid CY-2993, C-2234(C) PET, and E-2732 from the National Cancer Institute, Public Health Service, Bethesda, Md.

† Trade mark for 4-aminopteroylglutamic acid or 4-aminofolic acid.

amination was performed, and histological sections of the tumor and of various organs were prepared for study in a conventional manner.

Animals that survived for 90 to 150 days after inoculation were reinoculated with the tumor as described above. If the tumor killed the animal in the expected time it was assumed that the disease was cured by the original treatment.

Results

The control group. The survival in days after inoculation of 362 animals comprising the control groups for 34 consecutive experiments is shown as a cumulative frequency curve in FIGURE 1. In the entire group of 362 animals the mean survival was 15.4 ± 0.8 days, and the range varied from 12 to 20 days. In 34 consecutive experiments in which the control group contained from 9 to 13 animals, the mean survival time (MST) of the control groups varied from 13.5 to 17.0 days; the average MST was 15.3 ± 1.87 days.

The total white blood cell count varied from 5000/cu.mm. to 770,000/cu.mm. in individual animals on day 13 after inoculation. In individual experiments the mean total white blood cell count of the control group varied from 45,000/cu.mm. to 327,000/cu.mm. The mean of the mean total white blood cell count in 34 groups was 152.8 ± 68.4, and the white blood cell count on day 13 exceeded 30,000/cu.mm. in 93 per cent of animals in the control groups.

The effect of caloric restriction on the natural history of leukemia IRC741. This effect is summarized in TABLE 1. Animals in the control group were fed

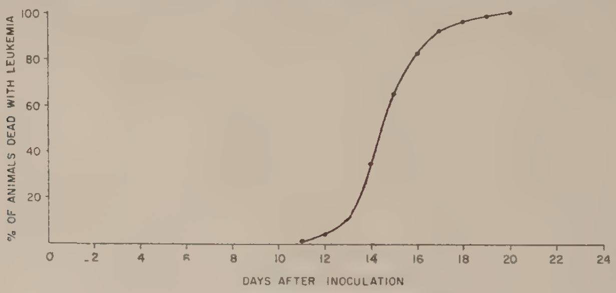

FIGURE 1. Day of death of Fischer rats inoculated with IRC741 leukemia.

TABLE 1
THE EFFECT OF CALORIC RESTRICTION ON THE PROGRESS OF THE DUNNING LEUKEMIA

Total dose mg./kg.	Daily dose* mg./kg.	Mean survival time (days)	Change in mean survival time (days)	Mean total WBC × 1000 day 13 Tr./con.	No. animals leukemic† on day 13 Tr./No.	No. animals leukemic† on day 13 Con./No.	Toxic deaths	Wt. change (gm.) Tr./Con.	No. cures‡ / No. Rx
66%*		18.3/17.5	0.8	89/149	10/10	9/9	0/10	19/35	0
50%*		19.6/17.5	2.1	45/149	7/10	9/9	0/10	9/35	0

* Per cent of intake of controls.
† Total WBC more than 30,000 cu.mm.
‡ Survival more than 100 days without evidence of leukemia.

TABLE 2

THE EFFECT OF CHLORAMBUCIL ON THE DUNNING LEUKEMIA

Total dose mg./kg./μM/kg.	Daily dose* mg./kg./μM/kg.	Mean survival time (days)	Change in mean survival time (days)	Mean total WBC × 1000 day 13 Tr./Con.	No. animals leukemic† on day 13		Toxic deaths	Wt. Change (gm.) Tr./Con.	No. cures‡/No. Rx
					Tr./No.	Con./No.			
1.4/5.8	0.1/0.42	15.9/16.1	−0.2	125/154	9/10	9/10	0	18/16	0
4.2/17.6	0.3/1.26	17.4/16.1	1.4	52/154	8/10	9/10	0	14/16	0
7.7/32.3	0.55/2.31	22.1/16.6	5.5	14/111	0/10	9/10	0	16/20	0
15.4/64	1.1/4.62	26.5/16.6	9.9	15/111	0/10	9/10	0	17/20	0
30.8/129	2.2/9.2	∞	∞	9/111	0/10	9/10	0	7/20	10/10
61.6/259	4.4/18.5	∞	∞	10/111	0/10	9/10	1/10	0/20	9/9
92.4/388	6.6/27.7	∞	∞	4/224	0/10	10/10	2/10	−18/16	8/8

* Daily I.P.

† Total WBC more than 30,000 cu.mm.

‡ Survival more than 100 days without evidence of leukemia.

Purina Fox Checkers* ad libitum, and total food consumption was determined each day. At the end of each day, animals in the experimental groups were offered (and consumed) 66 per cent (group A) and 50 per cent (group B) of the food consumed by animals in the control group.

The animals in group A gained an average of 19 gm. in 14 days (54 per cent of the average weight gained by animals in the control group). The mean survival time of animals in this group was 0.8 days greater than that of animals in the control group. The mean total white blood cell count of animals in group A was 89,000/cu.mm., while the mean total white blood cell count of animals in the control group was 149,000; experimental and control values are not significantly different ($p > .05$). The total white blood cell count on day 14 exceeded 30,000/cu.mm. for all animals in this group.

The average weight gained by animals in group B after 14 days (caloric intake restricted to 50 per cent of that of the control group) was 9 gm. (26 per cent of the average weight gained by animals in the control group). The mean survival time of animals in group B was 2.1 days greater than that of the control group, and the mean total white blood cell count was significantly less than that of the control group. Three animals in group B had a normal total white blood cell count and less than 10 per cent immature cells in the peripheral blood on day 14. All animals in both groups subsequently died of leukemia.

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* Manufactured by Purina Mills, Buffalo, N. Y.

Effect of Alkylating Agents

The effect of Chlorambucil [*CB 1348*; *p-N,N-di-(β-chloroethyl)aminophenylbutyric acid*]. The effects of various doses of Chlorambucil on leukemia IRC741 are summarized in TABLE 2, and the experimental design is illustrated in FIGURE 2. A daily dose of 0.3 mg./kg. produced an increase in mean survival time of 2.2 days and a reduction in the total white blood cell count on day 13. The treated animals gained as much weight as did the control group. Doses of 1.1 mg./kg./day produced a greater suppression of the disease without interference with weight gain, whereas a daily dose of 2.2 mg./kg. cured the disease in all animals. When the daily dose was increased to 6.6 mg./kg., marked weight loss resulted, and 2 of 10 animals succumbed to the drug (there was no evidence of the disease at the time of death); the 8 surviving animals were cured of the disease. A daily dose of 7.7 mg./kg./day killed all of the animals.

Three dose-response curves drawn from this experiment are shown in FIGURE 3. It is apparent that the increase in mean survival time varies in a linear fashion with the logarithm of the total dose in μM/kg. over a range of more than 15 μM/kg. When the total dose was increased from 64 to 129 μM/kg., the percentage of animals cured by the drug increased from 0 to 100 per cent.

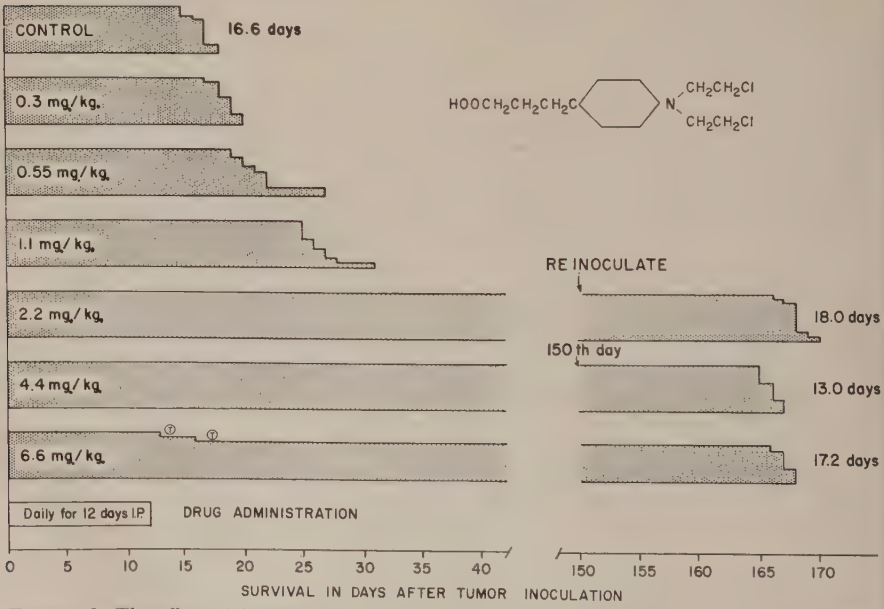

FIGURE 2. The effect of Chlorambucil on survival of Fischer rats with the Dunning leukemia.

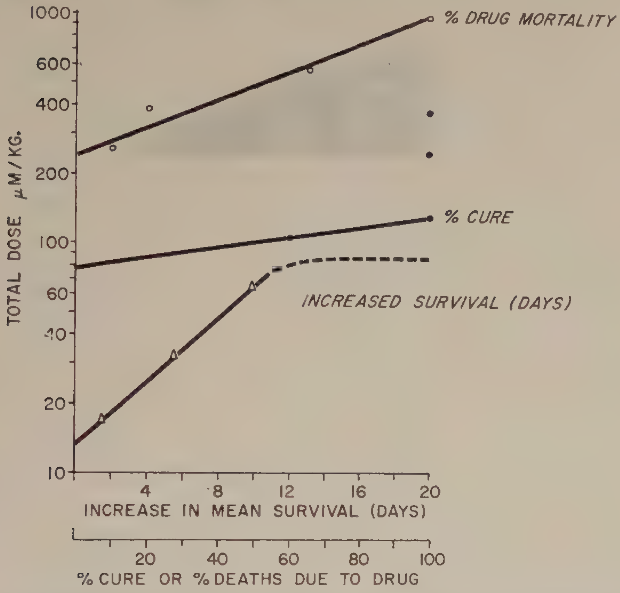

FIGURE 3. Dose-response curves of the effect of Chlorambucil in Fischer rats with the Dunning leukemia.

A total dose of 259 μM/kg. produced 10 per cent mortality, and a total dose of 457 μM/kg. produced 100 per cent mortality.

The effect of nitrogen mustard (methyl-bis-β-chloroethylamine). The effects of various doses of nitrogen mustard on leukemia IRC741 are summarized in TABLE 3. A total dose of 0.25 mg./kg., administered as a single dose of 0.05 mg./kg. every fourth day (beginning the day after inoculation), produced an increase in mean survival time of 2.1 days ($p < .01$), a reduction in mean total white blood cell count, and a reduction in the number of leukemic animals (as defined here) on day 14. A fivefold increment in dosage produced a marked increase in mean survival time and suppression of the leukemic process, but did not cure the disease in any animal; this dose also provoked marked weight loss with no toxic deaths. A sevenfold increment in dose (single dose, 0.35 mg./kg.) cured the disease in 1 animal out of 10. A tenfold increase (single dose, 0.5 mg./kg.) was fatal to 1 animal of the 10 tested, and cured the disease in all 9 animals that survived treatment. When the individual dose was increased to 0.6 mg./kg., all animals succumbed to the drug after 3 doses.

The three dose-response curves of this experiment are shown in FIGURE 4. Increase in mean survival time (in days) in treated animals varied in a linear fashion with the logarithm of the total dose in μM/kg.; 100 per cent cure occurred only with a dose that produced 10 per cent mortality.

The effect of Thio-TEPA (triethylenethiophosphoramide). The results of a study of the effect of Thio-TEPA on the Dunning leukemia are summarized in TABLE 4.

TABLE 3
THE EFFECT OF NITROGEN MUSTARD ON THE DUNNING LEUKEMIA

Total dose* mg./kg. µM/kg.	Daily dose mg /kg. µM/kg.	Mean survival time (days)	Change in mean survival time (days)	Mean total WBC × 1000 day 13 Tr /Con.	No. animals leukemic† on day 13		Toxic deaths	Wt. change (gm.) Tr./Con.	No cures‡ No. Rx
					Tr./No.	Con./No.			
0.25 1.60	0.05 0.32	18.2/16.1	2.1	44/146	4/10	9/10	0/10	20/17	0/10
0.5 3.20	0.10 0.64	26/18.7	7.3	19/79	1/10	7/10	0/10	3/12	0/10
0.75 4.80	0.15 0.96	30.2/18.7	11.5	22/79	2/10	7/10	0/10	—	0/10
1.0 6.40	0.20 1.28	31.7/18.7	13.0	20/79	1/10	7/10	0/10	−11/12	0/10
1.25 8.30	0.25 1.60	35.3/18.7	16.6	23/79	1/10	7/10	0/10	−19/12	0/10
1.75 11.20	0.35 2.24	35.6/18.8	17.8	16/59	0/10	8/10	0/10	−15/6	1/10
2.0 12.80	0.40 2.56	39.6/18.7	20.9	22/79	1/10	7/10	0/10	−22/12	2/10
2.5 15.5	0.50 3.10	∞	∞	19/59	0/10	8/10	1/10	−29/6	9/9
1.8 19.20	0.60 3.84	9.1/18.7	Tox.	—	-	—	10/10	−30/12	0

* Every fourth day × 5.
† Total WBC more than 30,000 cu.mm.
‡ Survival more than 100 days without evidence of leukemia.

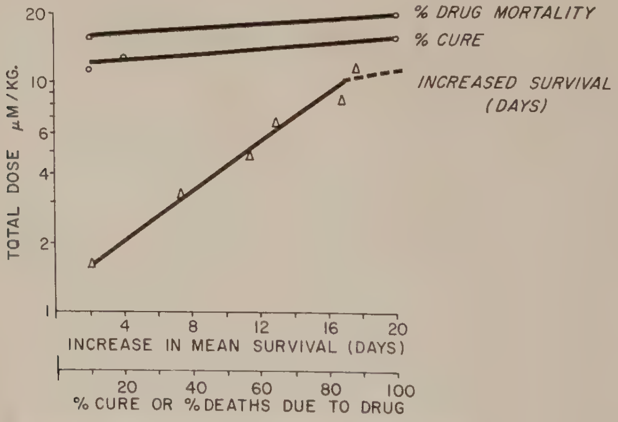

FIGURE 4. Dose-response curves of the effect of nitrogen mustard in Fischer rats with the Dunning leukemia.

TABLE 4

THE EFFECT OF THIO-TEPA ON THE DUNNING LEUKEMIA

Total dose* mg./kg μM/kg	Daily dose mg./kg. μM/kg.	Mean survival time (days)	Change in mean survival time (days)	Mean total WBC × 1000 day 13 Tr./Con.	No. Animals leukemic† on day 13		Toxic deaths	Wt. change (Gm.) Tr./Con.	No. cures‡ No. Rx
					Tr./No.	Con./No.			
0.35 1.855	0.05 0.265	15.9/16.1	—	150/146	10/10	9/10	0/10	19 3/17	0/10
0.875 4.62	0.125 0.66	16.3/16.1	0.2	95/146	10/10	9/10	0/10	22/17	0/10
1.75 9.31	0.25 1.33	23.3/18.9	4.4	15/145	0/10	10/10	0/10	8/9	0/10
2.94 15.61	0.42 2.23	32.4/19.0	13.4	10/53	0/10	8/10	0/10	4/14	0/10
7.0 37.1	1.0 5.3	36.4/16.1	20.3	16/145	1/20	18/20	2/20	−6/9	9/20
15.12 80.1	1.68§ 8.9	∞	∞	4/53	0/10	8/10	2/10	−10/14	8/8
14.0 74.2	2.0 10.6	∞	∞	8/145	0/10	10/10	6/10	−6/9	4/4
19.39 194	2.77 14.7	13.2/16.1	Tox.	—	—	—	10/10	−27/14	—
	3.35 17.8	8.6/16.1	Tox.	—	—	—	10/10	—	—
	6.7 35.6	5.9/16.1	Tox.	—	—	—	10/10	—	—

* Every other day × 7.

† Total WBC more than 30,000 cu.mm.

‡ Survival more than 100 days without evidence of leukemia.

§ Every other day × 9.

When given in a dose of 0.25 mg./kg. intraperitoneally every other day for 14 days, Thio-TEPA produced a significant increase in mean survival time (4.4 days) and delayed the appearance of leukemia, but all animals succumbed to the disease; 0.5 mg./kg. had no demonstrable effect. A dose of 1.0 mg./kg. every other day for 7 days produced weight loss and was fatal to 2 of 20 animals. The disease was cured in 9 of the 18 animals that survived treatment. A dose of 2.0 mg./kg. every other day for 7 days was fatal to 6 of 10 animals, but all survivors were cured. Dose-response curves in three parameters are plotted as the logarithm of the total dose in μM/kg. and are remarkably parallel; considerable overlap between mortality and cure is apparent (FIGURE 5).

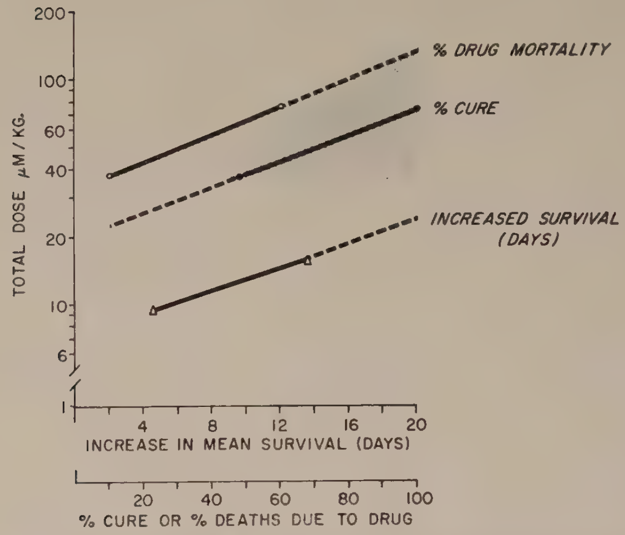

FIGURE 5. Dose-response curves of the effect of Thio-TEPA in Fischer rats with the Dunning leukemia.

TABLE 5

THE EFFECT OF MYLERAN ON THE DUNNING LEUKEMIA

Total dose*	Daily dose	Mean survival time (days)	Change in mean survival time (days)	Mean total WBC × 1000 day 13	No. Animals leukemic† on day 13		Toxic deaths	Wt. change (gm.)	No cures‡ / No. Rx
$\frac{\text{mg./kg.}}{\mu\text{M/kg.}}$	$\frac{\text{mg./kg.}}{\mu\text{M/kg.}}$			Tr./Con.	Tr./No.	Con./No.		Tr./Con.	
$\frac{4.5}{18}$	$\frac{0.5}{2.0}$	19.4/19.5	−0.1	48/44	7/10	6/10	0	7/5	0
$\frac{18}{72}$	$\frac{2.0}{8}$	19.2/19.5	−0.3	56/44	7/10	6/10	0	5/5	0
$\frac{27}{108}$	$\frac{3.0}{12}$	22.1/19.5	2.6	9/44	0/10	6/10	0	10/5	0
$\frac{36}{144}$	$\frac{4.0}{16}$	21.5/19.5	2.0	11/44	1/10	6/10	0	7/5	0
$\frac{45}{180}$	$\frac{5.0}{20}$	21.4/19.5	1.9	15/44	1/10	6/10	1/10	2/5	0
$\frac{59}{234}$	$\frac{6.6}{26}$	21.5/19.5	2.0	10/44	1/10	6/10	0/10	2/5	0
$\frac{72}{288}$	$\frac{8.0}{32}$	19.3/19.5	−0.2	10/44	0/10	6/10	2/10	5/5	0

* Every other day × 9.
† Total WBC more than 30,000 cu.mm.
‡ Survival more than 100 days without evidence of leukemia.

Table 6

The Effect of A-Methopterin on the Dunning Leukemia

Total dose* $\frac{\text{mg./kg.}}{\mu\text{M/kg.}}$	Daily dose mg./kg.	Mean survival time (days)	Change in mean survival time (days)	Mean total WBC × 1000 day 13 Tr./Con.	No. animals leukemic† on day 13 Tr./No.	Con./No.	Toxic deaths	Wt. change (gm.) Tr./Con.	$\frac{\text{No. cures‡}}{\text{No. Rx}}$
$\frac{0.40}{.91}$	0.05	16.6/16.9	−0.3	260/145	10/10	10/10	0/10	8/6	0/10
$\frac{0.80}{1.82}$	0.10	17.1/16.9	0.2	139/145	10/10	10/10	0/10	7/6	0/10
$\frac{1.20}{2.73}$	0.15	25.5/17.3	8.2	31/146	2/10	10/10	0	13/30	0
$\frac{2.4}{5.46}$	0.30	28.8/17.3	11.5	23/146	2/10	10/10	0	13/30	0
$\frac{4.96}{11.3}$	0.62	34.2/17.3	16.9	16/146	0/9	10/10	1/9	8/30	0
$\frac{10.00}{22.75}$	1.25	52/17.3	34.7	18/146	1/9	10/10	1/9	−3/30	4/8
$\frac{20.0}{45.50}$	2.5	Tox.	Tox.	8/146	0/9	10/10	8/9	−13/30	1/1

* Every other day × 9.
† Total WBC more than 30,000 cu.mm.
‡ Survival more than 100 days without evidence of leukemia.

The effect of Myleran (1,4-dimethanesulfonoxybutane). The results of a study of the effects of various doses of Myleran on the Dunning leukemia are summarized in TABLE 5.

Doses from 3.0 to 6.6 mg./kg. every other day for 9 doses produced a marked reduction in the mean total white blood cell count, but there was only a slight increase in mean survival time. There was little effect on the growth of the tumor at the site of implantation (data not shown here).

*A-Methopterin (Methotrexate; 4-amino-*N^{10}*-methylpteroylglutamic acid).* The results of a study of the effects of various doses of A-Methopterin on the Dunning leukemia are summarized in TABLE 6.

A dose of 0.1 mg./kg. every other day for 8 doses had no demonstrable effect on the disease. In contrast, a dose of 0.15 mg./kg. every other day for 8 doses produced an increase of 8.2 days in mean survival time, but all treated animals died of the disease. When the dose was increased to 1.25 mg./kg. every other day for 8 doses there was weight loss and 1 toxic death; the disease was cured in 4 of the 8 animals that survived treatment (TABLE 6). Eight of 9 animals treated with 2.5 mg./kg. of A-Methopterin succumbed to this drug, but the animal that survived treatment was cured. These data are graphically presented in FIGURE 6.

6-Mercaptopurine. Intraperitoneal doses of 5 mg./kg. every other day for 5 doses had no significant effect on the course of the disease, but 7 mg./kg. every other day for 5 doses evoked a significant increase in mean survival time and a reduction of the mean total white blood cell count on day 13. Doses of 15 mg./kg. every other day for 7 days produced approximately the same degree of suppression of the disease as doses of 9 mg./kg. daily for 12 days, suggesting that therapeutic effect may be a function of total dose. Similarly, the effect of 30 mg./kg. every other day for 7 days was approximately as strong as 17 mg./kg. daily for twelve days (TABLE 7).

Only one animal was cured of the disease in the entire study. This rat received 24 mg./kg. daily for 12 days, a dose that produced marked weight loss and one toxic death. A dosage regimen that produces 100 per cent mortality has not yet been studied.

Urethan. The results of a study of the effect of urethan on the Dunning leukemia are summarized in TABLE 8.

Intraperitoneal administration of 50 mg./kg. for 11 days had no demonstrable effect on the disease, whereas daily doses of 100 mg./kg. produced slight prolongation of life and delay in the appearance of leukemia. Daily doses

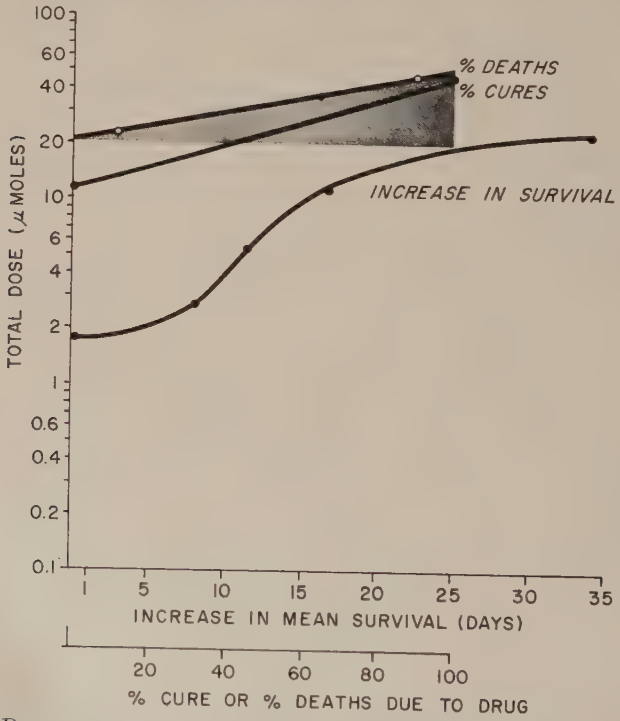

FIGURE 6. Dose-response curves of the effect of A-Methopterin in Fischer rats with the Dunning leukemia.

TABLE 7

THE EFFECT OF 6-MERCAPTOPURINE ON THE DUNNING LEUKEMIA

Total dose[*] mg./kg. µM/kg.	Daily dose mg./kg. µM/kg.	Mean survival time (days)	Change in mean survival time (days)	Mean total WBC × 1000 day 13 Tr./Con.	No. animals leukemic[‡] on day 13		Toxic deaths	Wt. change (gm.) Tr./Con.	No. cures[§] No. Rx
					Tr./No.	Con./No.			
25 162	5 32.3	15.8/14.5	1.3	121/179	8/9	9/10	0/9	7/8	0/9
35 226	7 45	16.7/14.5	2.2	106/179	6/9	9/10	0/9	9/8	0/9
105 710	15 97	25.3/15.9	8.4	16/145	1/10	10/10	0/10	−3/5	0/10
210 1360	30 19.4	25.6/15.9	9.7	12/145	0/10	10/10	1/10	−9/5	0/10
108 700	9[†] 58.1	24.5/16.9	7.6	12/87	0/10	10/10	0/10	−4/3	0/10
156 1010	13 84	33.8/18.6	15.2	16/52	0/10	7/10	0/10	−8/10	0/10
204 1320	17 110	26.2/16.9	9.3	14/87	0/10	10/10	0/10	−8/3	0/10
288 1860	24 155	35.5/18.6	16.9	10/52	0/10	7/10	1/10	−21/0	1/10
360 2325.6	30 193.8	Tox.	Tox.	7/69	0/6	9/11	5/6	−2/26	
360 2325.6	30 193.8	Tox.	Tox.	5/165	0/2	14/15	5/6	−2/12	1/6

[*] Every other day × 5.
[†] Daily × 12.
[‡] WBC more than 30,000 cu.mm.
[§] Survival more than 100 days without evidence of leukemia.

of 400 mg./kg. produced greater prolongation of life, with little evidence of toxicity, but daily doses of 500 mg./kg. caused weight loss and 2 toxic deaths in a group of 10 animals. The 8 animals that survived treatment all succumbed to the disease.

In these studies there was no evidence that urethan has curative activity. This drug appeared to retard the development of leukemia and dissemination of the disease, but there was little effect on the growth of the subcutaneous tumor at the site of inoculation of the tumor. Further work on this observation is in progress.

TABLE 8

THE EFFECT OF URETHAN ON THE DUNNING LEUKEMIA

Total dose* mg./kg.	Daily dose mg./kg.	Mean survival time (days)	Change in mean survival time (days)	Mean total WBC × 1000 day 13	No. animals leukemic† on day 13		Toxic deaths	Wt. change (gm.)	No. cures‡ / No. Rx
				Tr./Con.	Tr./No.	Con./No.		Tr./Con.	
550	50	17.4/16.9	0.5	46/65	6/10	7/10	0/10	20/21	0/10
1100	100	15.5/13.5	2.0	38/169	7/10	10/10	0/10	20/9	0/10
2200	200	18.4/13.5	4.9	22/169	3/10	10/10	0/10	6/9	0/10
3300	300	19.8/13.5	6.3	26/169	3/10	10/10	0/10	6/9	0/10
4400	400	20.3/13.5	6.8	24/169	3/10	10/10	0/10	4/9	0/10
5.5 gm.	500	21/14.7	6.3	16/182	1/8	8/10	2/10	−8/8	0/8

* Daily × 11.

† WBC total more than 30,000 cu.mm.

‡ Survival more than 100 days without evidence of leukemia.

Discussion

The most useful index of therapeutic activity against the Dunning leukemia is prolongation of life, as measured by increased survival time. Mean total white blood cell counts and the number of animals with total white blood cell counts exceeding 30,000/cu.mm. are helpful, but more variable, criteria. Demonstration of malignant cells in the peripheral blood serves as an indication of dissemination and progression of the disease.

The rate of progression of the disease may be diminished by caloric restriction, so that this must be taken into account in evaluating the effect of toxic chemicals that interfere with appetite. The studies reported here that deal with caloric restriction are being extended, and the results will be reported later.

The fact that this disease can be cured by a spectrum of chemotherapeutic agents makes it particularly valuable for quantitative pharmacological studies. Cure, as defined here, is an absolute and constant value, which makes it possible to compare chemotherapeutic agents or various doses of the same material with a degree of accuracy that can hardly be achieved where the parameter measured is merely increase in survival time or in tumor weight or size. The use of this disease makes it possible to study the mechanisms by which a chemotherapeutic agent suppresses the disease, cures the disease, or is fatal to the host. Careful quantitative study of the relationship of dose to each of these phenomena can provide evidence to indicate whether the mechanism that suppresses the disease is the same as, or different from, the mechanism by which the disease is cured. Similarly, the cause of death of the host can be compared, by quantitative pharmacological methods, with the mechanisms that produce suppression and cure. Since the ultimate objective of research in cancer chemotherapy is cure, it is satisfying to the investigator to work with a form of the disease in which complete cures can be achieved.

Analysis of the dose-response curves presented for each agent permits the

TABLE 9

THE EFFECT OF VARIOUS CANCER CHEMOTHERAPEUTIC AGENTS ON THE SURVIVAL OF FISCHER RATS WITH THE DUNNING LEUKEMIA

Agent	Minimum effective dose* (μM/kg.)	Minimum lethal dose† (μM/kg.)	$\frac{\text{MLD}}{\text{MED}}$
Nitrogen mustard	1.6	15.5	10
Thio-TEPA	9	37.5	4
CB1348	18	259	15
Myleran	108	180	1.7
Urethan	1220	6100	5
6-Mercaptopurine	226	1860	8
A-Methopterin	1.8	20	11.1

* Total dose producing 2-day increase in mean survival.
† Total dose producing 10 to 20 per cent mortality.

calculation of the standard pharmacological values, the minimum effective dose, the minimum curative dose, the minimum lethal dose, and the determination of the indices derived from these values that are helpful in comparing relative effectiveness and safety of related drugs (see TABLE 9).

The ratio of the minimum lethal dose to the minimum effective dose achieved with nitrogen mustard is 10, indicating that this is a highly effective agent with a good margin of safety between the effective and the toxic doses. Thio-TEPA is less active than nitrogen mustard (on a molar basis) and less toxic, but the margin between the effective and the toxic doses is less than with nitrogen mustard. This would indicate that, in this system at least, nitrogen mustard is superior to Thio-TEPA. Chlorambucil is less active and less toxic than nitrogen mustard, and the margin of safety between the effective and the lethal doses is greater than with nitrogen mustard. These observations probably parallel the results of clinical experience with these three agents, but quantitative data in man are not available for precise comparison.

The relationship of the minimum curative dose and the minimum lethal dose of these agents is shown in TABLE 10. With all of the agents that exhibited curative activity, the ratio of the curative dose to the lethal dose was quite

TABLE 10

THE EFFECT OF VARIOUS CANCER CHEMOTHERAPEUTIC AGENTS ON THE SURVIVAL OF FISCHER RATS WITH THE DUNNING LEUKEMIA

Agent	Minimum curative dose* (μM/kg.)	Minimum lethal dose† (μM/kg.)	$\frac{\text{MLD}}{\text{MCD}}$
Nitrogen mustard	11.2	15.5	1.4
Thio-TEPA	25	37.5	1.5
CB1348	80	259	3.2
Myleran	—	180	—
Urethan	—	6100	—
6-Mercaptopurine	1860	1860	1.0
A-Methopterin	13	20	1.5

* Total dose producing 10 to 20 per cent cures.
† Total dose producing 10 to 20 per cent mortality

small. Chlorambucil may be superior to the other agents shown here. It is apparent that there is much room for improvement in these drugs, and one would hope that with new agents the margin between the minimum curative dose and the minimum lethal dose may be increased. It would seem that such quantitative comparisons of newer effective agents might provide a reasonable basis for selection of new agents for clinical trial and for aiding the direction of synthesis of new agents.

The very minimal effect of Myleran on this leukemia probably contributes important evidence as to the origin and nature of the disease. The immature cells in the peripheral blood seem morphologically to resemble myeloblasts and promyelocytes or immature monocytes more closely than immature lymphocytes. The sensitivity of this leukemia to Chlorambucil and its insensitivity to Myleran suggest that the disease is more likely to be of lymphoid origin.

The fact that this disease also responds reproducibly and well to 6-Mercaptopurine, A-Methopterin, and urethan indicates that it will be useful for quantitative comparison of new candidate compounds.

The use of very highly inbred rats for studies of cancer and cancer chemotherapeutic agents undoubtedly has all of the same virtues that have so often been proclaimed for similar work in mice.

Summary

The Dunning leukemia (IRC741) in Fischer line 344 rats has been found to be useful for quantitative pharmacological studies of cancer chemotherapeutic agents. The biological uniformity of the disease permits rapid and accurate evaluation of therapeutic activity. The disease is cured by nitrogen mustard, Thio-TEPA, Chlorambucil, A-Methopterin, and 6-mercaptopurine. It is suppressed, but not cured, by urethan, and is little affected by Myleran. The fact that the disease may be cured by some agents provides an additional valuable parameter for quantitative comparison of related compounds and for comparison of new agents with drugs of known activity.

Reference

1. Dunning, W. F. & M. R. Curtis. 1957. A transplantable acute leukemia in an inbred line of rats. J. Natl. Cancer Inst. **19:** 845.

STUDIES OF THE CULTURE OF LEUKEMIC CELLS *IN VITRO*[*]

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With the exception of a mouse leukemic cell line isolated by DeBruyn and described in 1955,[1] it has not been possible to propagate leukemic cells continuously in the absence of a feeder layer of nonleukemic cells. In 1938 Bichel[2] reported that leukemic cells from mice would not reproduce in the culture media used at that time, but required a feeder layer of chick fibroblasts. These studies were confirmed and extended by DeBruyn *et al.*,[3] who grew leukemic lymphoblasts on a supporting layer of mouse fibroblasts.

In this paper I shall describe the successful culture of several strains of L-5178, a lymphocytic mouse leukemia. The medium developed, which contains peptone and very high levels of folic acid, permits the continuous reproduction, *in vitro* and in the absence of feeder layers, of the leukemic cells.† In support of the statement that leukemic cells representative of L-5178 reproduce in the medium devised, several lines of evidence have been obtained. Titration studies in susceptible strains of mice with cells grown in culture have demonstrated that there is no loss of virulence on long-continued propagation *in vitro*. The cells in culture retain their morphologic characteristics unaltered, continue to grow in the supernatant fluid of the medium, and retain their round-cell character and staining properties. Obtained from the ascitic cavity of mice during the logarithmic phase of their growth, cultures resume logarithmic rates of reproduction with an intervening lag period of no more than 5 hours. In addition, cytological studies have demonstrated that the diploid character of the cells is retained after 60 cell generations in culture.[4] Finally, single cells, isolated from populations grown in culture, have given rise to genetically homogeneous populations of cells with the same general properties as those exhibited by the parent strains.[5]

The medium used is presented in TABLES 1, 2, and 3. The defined components are essentially those of a medium described by Scherrer;[6] however, toxic as well as nonessential components have been omitted. Two alterations, essential for the growth of the leukemic cells, were necessary: one, a high level of folic acid, to satisfy an unusually high requirement for this vitamin,[7] and, two, the presence of a peptone, since reproduction of the leukemic cells is acutely dependent on one or more substances in such materials. Work concerning the isolation and characterization of the growth factors of peptone is in progress.[8] Other additions to the modified medium of Scherrer are glutamine, ascorbic acid, and glutathione. It is unlikely that each of the required metabolites is present as yet at an optimal level, for emphasis has been

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† Supplied by L. W. Law of the National Cancer Institute, Public Health Service, Bethesda, Md.

TABLE 1

CONSTITUENTS OF CULTURE MEDIUM FOR L-5178 LYMPHOBLASTS

	Medium concentration
	(gm./l.)
A. NaCl	8.0
KCl	0.4
$MgCl_2 \cdot 6H_2O$	0.1
Na_2HPO_4	0.06
$NaH_2PO_4 \cdot H_2O$	0.067
B. Glucose	1.0
C. $NaHCO_3$	1.0
D. 5% acid hydrolyzed casein + tryptophan, 0.5 mg./ml.	5 ml./l.
	(mg./l.)
E. Glycine	20
Cystine	7.5
Histidine	20
F Glutamine	200
G. Vitamins:	
Thiamin·HCl	1.0
Nicotinamide	0.5
Ca-Pantothenate	0.5
Pyridoxal·HCl	0.5
D-Ribose	0.5
Riboflavin	0.5
Choline chloride	1.5
i-Inositol	1.5
H. Biotin	0.01
I. Ascorbic acid	1.6
Glutathione (reduced)	1.5
J. Serum	2–10%
K. Peptone	0.06%
L. Folic acid	10 mg./l.
M. Penicillin	100 units/ml.
Streptomycin	0.050 mg./ml.
O. Phenol red	10 mg./l.
P. $CaCl_2 \cdot H_2O$	0.182 gm./l.

placed on the elimination from the medium of nonessential metabolites and the replacement of the peptone by compounds of known composition.

Obtained from the peritoneal cavity of DBA/2 mice, the cells were cultured in 1 ml. of freshly prepared medium. Following gentle centrifugation of the cultures for 90 sec., and aspiration of 0.9 ml. of the supernatant fluid, 0.9 ml. of freshly prepared medium was added daily. Following 4 successive transplants of supernatant fluids (uncentrifuged), the cultures were free of histiocytes, since the latter cells remained attached to the glass. The extent of growth of histiocyte-free cultures was determined by hemocytometer counts; subcultures were made every 1 to 4 days by transferring aliquots to fresh media. In order to prove that growth of the leukemic cells occurred, cells from culture were titrated in C57/DBA/2 mice, using as a criterion the inverse relationship between survival time and number of cells injected. FIGURE 1 illustrates the results of one of three such experiments. Following each successive seventh doubling of the number of cells in culture, aliquots were reinjected into mice,

TABLE 2
STOCK CONCENTRATIONS, STERILIZATION PROCEDURES, AND STORAGE OF CONSTITUENTS OF THE MEDIUM

Stock code*	Stock concentration	Sterilization procedure	Storage condition (°C.)
A	10×	Autoclave	25
B	20×	Filter†	25
C	75×	Filter	0
D	200×	Filter	0
E	15×	Filter	25
F	100×	Filter	−16
G	50×	Filter	−16
H	100×	Filter	−16
I	$10^3\times$	Filter	−16
J	—	Filter	0
K	100×	Filter	0
L	200×	Filter	0
M	$10^3\times$	Filter	−16
O	500×	Filter	0
P	$10^3\times$	Filter	0

* See TABLE 1 for constituents of culture media.

† Sintered glass filters (Morton filters).

Riboflavin—0.1 N Na_2CO_3 to dissolve.

Biotin—0.1 N Na_2CO_3 to dissolve.

Cystine—0.1 N HCl to bring into solution: neutralize with 1 N NaOH and add to glycine and histidine.

Peptone—Prepare 10% solution (obtained from Walker Laboratories, Mt. Vernon, N. Y.) by heating at 100° C. for 20 min., store at 0° C. for 2 weeks, sterile filter, and dilute to 6 per cent.

Vitamin stocks are prepared each 60-day period.

Serum is dialyzed by stirring at 0° C. with 9 changes of 10 volumes of distilled water in a 72-hour period. The volume of the serum is reduced with air currents at 0° C. Volume is restored by the addition of NaCl solutions to obtain a concentration of 0.9 per cent and, following centrifugation at 10,000 g, is sterile filtered.

TABLE 3
PREPARATION OF THE MEDIUM

Stock solution*	Milliliters of stock solution
A	100.0
B	50.0
C	13.0
G	20.0
H	10.0
L	5.0
Demineralized H_2O	500.0
D	5.0
E	67.0
F	10.0
O	2.0
I	1.0
M	1.0
J	20–100
K	10
Demineralized H_2O	to 1 liter
Sterile filter	
P	1.0

* See TABLE 1 for constituents of culture media.

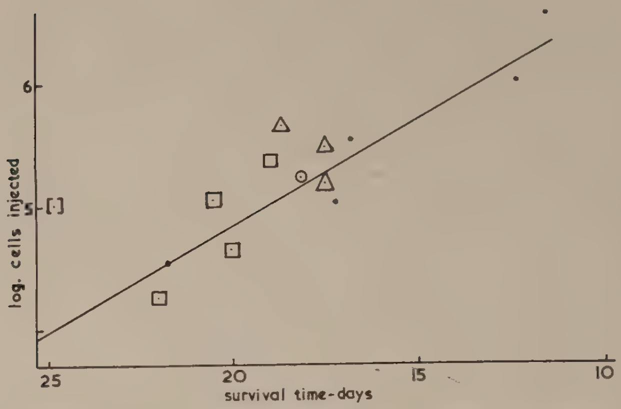

FIGURE 1. Titration of L-5178 lymphoblasts in DBA/2 mice. Symbols: ● = average survival time of five mice injected with the indicated number of tumor cells obtained directly from the mouse; △ = seventh generation in culture; ○ = fourteenth generation in culture; □ = twenty-first generation in culture.

using 2 animals for each point in the figure. All animals were subjected to autopsy. No significant difference was seen between the leukemias produced from cells grown in culture and those from mice. These results demonstrate that there is no loss of virulence during the period of leukemic cell multiplication in culture. The additional demonstration that the doubling time of the cells, free of all feeder cells, did not decrease over a period of 20 consecutive cell generations indicates that all the nutritional requirements of the leukemic cells are supplied by the culture medium. Similar observations have been made on pure lines (descendants of single leukemic cells) after 150 consecutive cell generations. Generation times of 12 to 20 hours were obtained while in agitated cultures and, in a specially devised medium, generation times of 10 to 14 hours were attained. FIGURE 2 illustrates the leukemic cells as they appear when obtained from culture.

Since growth of these cells is in the supernatant portion of the medium, convenient microbiological methods have been applied in order to determine responses in growth with repeated observations on the growth of individual cultures being made. The density of cell populations was determined by nephelometry.* Cells for the inoculum were taken from stock cultures in the logarithmic phase of growth and added to a sufficient volume of incubation medium to give a final concentration of cells of 2 to 3 × 10^5 cells/ml.; of this suspension, 4.5 ml. aliquots were placed in screw-cap culture tubes (16 × 125 mm.). Acid production was directly (but not accurately) related to growth; approximate growth responses were estimated by measuring the absorptive capacity of the supernatant incubation medium at 525 mμ near the absorption maximum of alkaline solutions of phenol red (present in the medium). However, direct determination of cell densities by hemocytometer counts remains the method of choice, since disruption of cells, as occurs with A-Methopterin,

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* A Coleman Model 9 Nephocolorimeter was used.

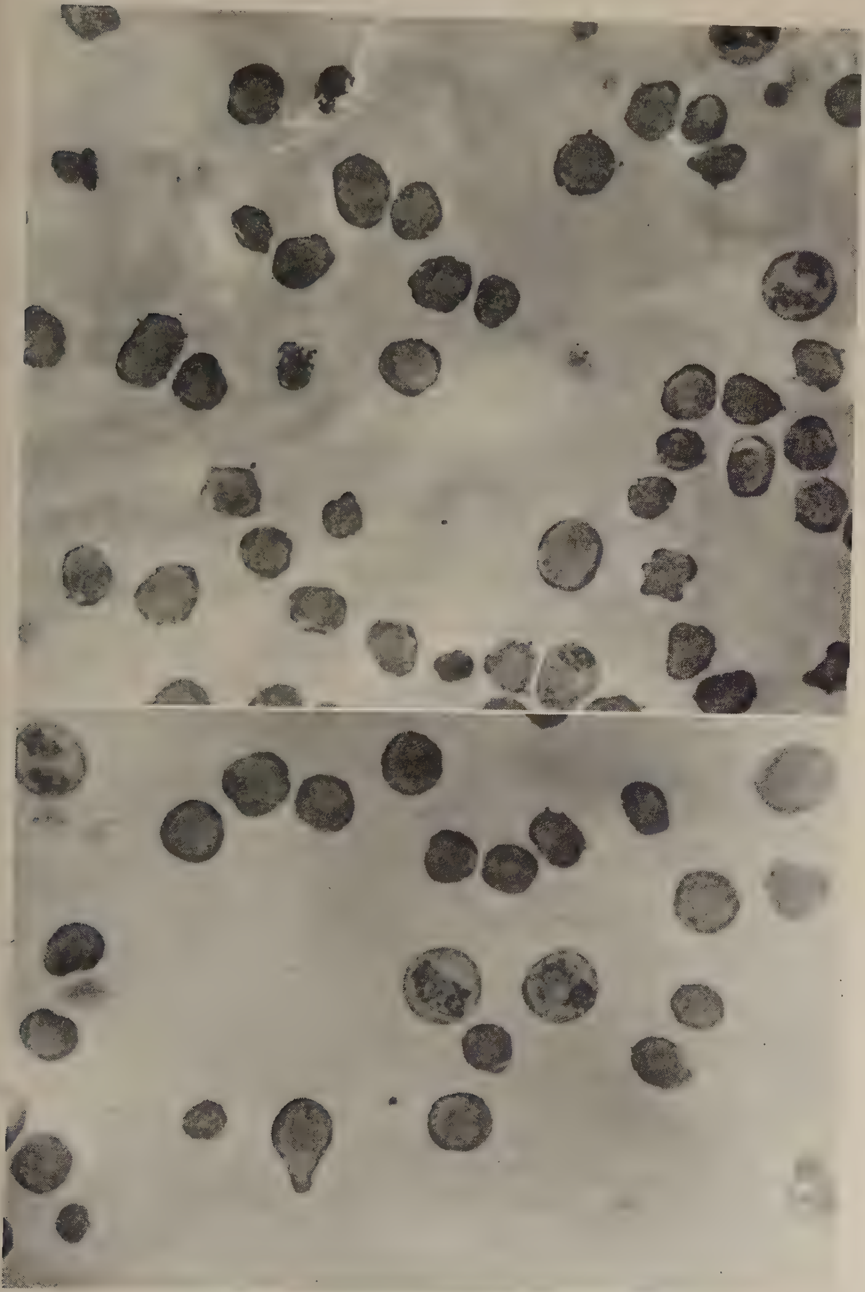

Figure 2. L-5178 lymphoblasts obtained from culture.

or drug-decomposition resulting in particles, as with 6-selenopurine, can yield spurious values in nephelometry.

Since the culture techniques were developed in order to provide a system for genetic, chemotherapeutic, and biochemical investigations of somatic mammalian tumor cells, studies of the growth responses of the tumor cells in the presence of known anticancer agents have been initiated. The sensitivity of the malignant lymphoblasts to known anticancer agents in culture (using 6 per cent dialyzed horse serum, 0.06 per cent peptone, and folic acid) is equivalent to and, in some cases, greater than the sensitivity of a number of other mammalian cell lines, as reported by Eagle and Foley.[9] The peptone used greatly reduces the toxicity of several chemotherapeutic agents for the leukemic cells. To produce 50 per cent inhibition of the growth of the cells in a medium containing 0.5 per cent peptone and 0.01 μg. (0.023 μmole) of folic acid/ml., 30 μmole of A-Methopterin/ml. are required while, in a medium with reduced levels of peptone (0.06 per cent) and high levels of folic acid [10 μg. (0.023 μmole)/ml.], only 0.007 μmole of A-Methopterin/ml. produces a comparable degree of inhibition. Since marked increases of toxicity have been observed with carcinostatic agents following simplification of the medium, comparisons of drug toxicities with those observed in the diverse systems described by Eagle and Foley appear to be unwarranted.

Of considerable interest has been the lack of toxicity of 6-azauracil to cells of S-180[10] or of L-5178-Y (a pure line) in culture, although in mice azauracil inhibits the growth of neoplasms derived from these tumor cells. However, azauridine, the riboside of azauracil, exhibits significant toxicity to cells of S-180 (at 121 μmole/ml.) and of L-5178-Y (at 2.4 μmole/ml.) in culture. Studies in mice have indicated that, at effective levels of dosage, about 5 per cent of administered azauracil is converted to ribose-containing derivatives, as reflected by the urinary excretion of the metabolically very stable riboside (azauridine). In agreement with this observation is the finding in mice that equivalent carcinostasis is obtained with azauridine in doses approximately one twentieth those required with azauracil.[11] That the inactivity of azauracil in culture indeed may be attributed to the inability of the tumor cells to form ribose-containing derivatives of azauracil, a function performed by the liver or other normal tissues of the host, is indicated by studies using S 180 cells and isotopically labeled azauracil, azauridine, and various labeled metabolites.[12]

Discussion

The inability of standard culture media to support the continuous multiplication of L-5178 lymphoblasts is attributable in part to the low levels of folic acid they provide. However, when grown in the presence of a feeder layer of histiocytes, or of embryonic chick fibroblasts, the leukemic cells will reproduce in media containing conventional levels of folic acid (0.01 to 0.1 μg./ml. of medium). In culture, histiocytes attach to the glass and, under these conditions, most typically resemble fibroblasts, although many other forms are seen, particularly in cultures with very long doubling times. Mixed cultures of histiocytes and L-5178 tumor cells have been maintained for a period of 45 days with reproduction of both leukemic cells and histiocytes. These mixed cultures produce the characteristic leukemia when reinjected into mice, although the culture is composed predominantly of histiocytes. Since as few as ten L-5178-Y cells will produce leukemia when injected into mice of the strain of origin, that is, DBA/2, this finding was not unexpected. When removed from the histiocyte feeder layer and cultured in the deficient medium, the leukemic cells die rapidly. However, when the deficient medium is supplemented with high levels of folic acid (10 μg./ml. of medium) and with peptone (0.06 per cent), the leukemic cells, freed of all nonleukemic cells, will reproduce with a constant generation time for at least one hundred and fifty consecutive cell generations. These results demonstrate that, in the nutrition of the leukemic cells, the feeder layer can be replaced by folic acid and peptone, and they suggest that the function of the feeder layer is to supply compounds otherwise obtainable only in the presence of high levels of folic acid and of unknown nutrients contained in peptone.

A second variety of leukemic cells, composed exclusively of P-815 mast cells,[13] has a similar high requirement for folic acid[14] and was initially grown in pure culture in the medium developed for the lymphoblasts. As with cultures of L-5178-Y, the morphologic, biochemical, and biological properties examined have remained unaltered in cultures of the P-815 leukemia in a special medium developed.[15] Although leukemic cells that reproduce in media containing conventional levels of folic acid have been reported,[16] their descriptions closely resemble those of mixed cultures described by Bichel[2] and DeBruyn *et al.*[3] as well as the mixed cultures of L-5178 cells and histiocytes grown in media deficient in the metabolites required by the leukemic cells.

Media devised for the culture of the lymphoblasts have not supported the continuous multiplication of other malignant or normal leukocytes, with the possible exception of monocytes and histiocytes. Of four mouse leukemic cell lines examined, only the two described have maintained constant rates of reproduction in culture. Also, representative blast cells obtained from human marrow thus far have failed to reproduce continuously in the present leukemic cell medium. Indeed, there are substantial differences between the nutritional requirements of the two leukemic lines that have been grown. Thus the mast cell line requires an unknown component of undialyzed horse serum, while the leukemic lymphoblasts have no apparent requirement for this factor; the latter cells require one or more factors supplied by peptones, while the mast cells, at least in the presence of undialyzed serum, do not require components of peptone.

Acknowledgments

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Discussion of the Paper

Lloyd W. Law (*National Cancer Institute, Public Health Service, Bethesda, Md.*): I had hoped Fischer would say something about the chromosomes in the L-5178 growing in suspension.

Fischer: The L-5178 cells in culture or in mice remain stable as diploid populations, in contrast to normal monkey kidney cell cultures. However, it may be characteristic of leukemic cells to be stable as diploids in any situation.

Law: I do not think that is true, but this is certainly interesting as far as L-5178 is concerned.

Question: Is it evident that normal cells will grow?

Fischer: No, it is not.

Part V. Hormone-Responsive Tumors

SCREENING TECHNIQUES AND PROBLEMS OF HORMONE-RESPONSIVE TUMORS*

By Jacob Furth and Kelly H. Clifton

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In introducing Part V on screening substances of potential value in control of tumors related to endocrine systems, we are not so much reporters of work done as mere theoreticians. There is little to report; the only extensive study in this area is that of Huggins *et al.*[1-3] with mammary fibroadenomas of the rat.

For a comprehensive survey of what is known on hormonal regulation of tumor growth we must turn to the recent reviews of Noble,[4] Kirschbaum,[5] Huggins,[6] and Currie and Illingworth.[7]

A systematic study of potential inhibitors of endocrines and endocrine-dependent tumors is long overdue. Currently, experimental chemotherapy aims at interference with growth of cells in general in hope of finding substances that selectively inhibit neoplastic cells. This will remain an arduous task until the basic difference between neoplastic and normal cells is known. Only then will it be possible to design rational experiments in the search for tumor inhibitors. This is not said to minimize such efforts. The work itself with inhibitors of nucleoprotein synthesis and growth in general, using neoplastic and normal cells, can yield information on the fundamental difference between the two cell types. As to the results, witness the control of chorioepithelioma obtained recently by Hertz[8] with folic acid antagonist.

In the field of endocrines, however, specific stimulants and inhibitors of cells are known. The target is limited to specific acceptors of cells. Some endocrines and their target organs, such as the breast and prostate, are not essential for life, so that their complete destruction or inhibition is without serious consequence. In other situations compensation can be attained by hormonal therapy. The influential substances are not species-specific, and the information obtained in animals is likely to be valid in man.

Most, if not all, current screening presupposes that the point of attack should be the neoplastic cell. Research in the domain of endocrines clearly indicates that neoplasms can arise by mere derangement of physiological forces that regulate the numbers of each cell type at an approximate level, without any extrinsic carcinogens or a virus. The growth of the tumors so induced is at first dependent on the persistence of the disturbance that created it.

FIGURE 1 is an oversimplified schematic presentation of the maintenance of the numbers of a cell type by a feedback mechanism of stimulating and restraining forces. Depression of one leads to hyperfunction of the other.

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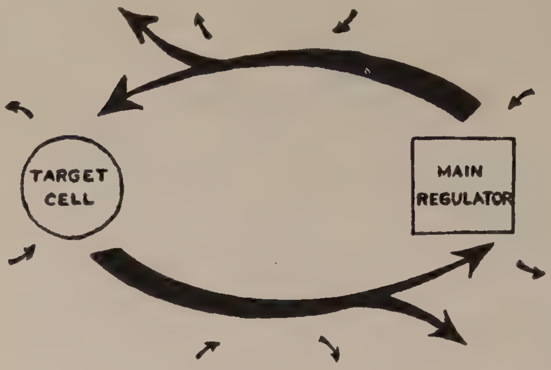

	Alteration	
Pathogenesis of neoplasia	Site	Character
1. Sustained deficiency of restraining force	Host	Somatic
2. Sustained excess of stimulating force	Host	Somatic
3. Altered responsiveness of target or regulator	Cell	Cytogenic

FIGURE 1. Scheme of feedback regulation.

There are secondary influences on both the regulator and the target organ, making this relationship fairly intricate. Tumors can arise either by sustained overstimulation or by reduction of the restraining force, as well as by alteration in cells.

Different types of tumors call for different approaches in therapy. When host factors are deranged, therapy aims at correction of the change in the host. When the cell is altered, the target of therapy will be the cell. Thus, redefinition of neoplasia is more than an academic matter. We proposed the following: neoplasia is a state of apparently unrestrained growth of cells caused either by permanent alteration in the cells or by extracytogenic forces, such as feedback derangement, caused by hormones[9] or viruses.[10]

Autonomy is best conceived as an irreversible alteration in the cell, whether due to a somatic mutation or to abnormal differentiation. In the case of endocrine tumors, knowledge of the deficiency may lead to specific measures of control; for example, loss of ability for hydroxylation at position 21 in adrenal steroidogenesis will cause an excess of adrenal cortex-stimulating hormone and secondary adrenal tumors correctable by specific inhibition of adrenotropic hormone with glucocorticoids.[11]

Until recently, hormonal derangements were the only conditioning factors known. Some viruses also can be conceived as conditioning agents. Intra-

TABLE 1

EXPERIMENTAL ENDOCRINE-RELATED TUMORS

Organ	Cell type	Potential screening value
Thyroid	Epithelial	Thyroid carcinoma
Pituitary	Thyrotrope	Thyroid carcinoma
Pituitary	Mammotrope	Breast carcinoma
Breast	Epithelial-fibroblastic	Breast carcinoma

Chiefly of theoretical interest: ovary, testis, pituitary (AtT),* adrenal cortex.
Unexplored: prostate,* kidney, lymphoma,* cervix.

* All but these are hormone induced.

cellular parasitism may interfere with the specific stimulating or restraining forces on cellular level.[10] Accordingly, it seems now that altered responsiveness can be due to diverse causes, and at least three causes can be assumed to exist: somatic mutation, abnormal differentiation, and extraneous self-replicating particles in cells. Although the latter have not been shown to be of importance in man, in view of our ignorance of the causes and of the nature of human cancers, we must consider biological analogies. Different types of intracellular events leading to apparently uncontrolled growth call for different experimental therapeutic approaches.

It has been demonstrated in numerous endocrine systems that the formation of a dependent tumor precedes that of an autonomous mutant. Extensive experience led us to conclude that neoplasms induced by hormonal derangements are likely to go through a phase of complete dependency, whereas those induced by carcinogens, such as radiation, are likely to be autonomous. However, even these autonomous tumors are at first highly responsive to their physiological regulators. Recently we have assayed more than twenty radiation-induced pituitary tumors.[12] All were autonomous, but none failed to respond, at least to some extent, to its physiological regulator. Such tumors are best called autonomous-responsive.

TABLE 1 lists experimental tumors that can be induced readily and are of potential screening value. Most valuable and ready for exploration are those tumors induced by sustained hormonal excess or deficiency, in which the stages of transformation from dependent to autonomous variants can be followed and the varying grades of malignancy and dependency in the variants can be preserved in the frozen state and made available when wanted.

The most important human neoplasms under strong endocrine influence are carcinoma of the breast, of the prostate, of the thyroid and, possibly, of the adrenal and uterus, and the lymphomas. Foremost among these is mammary carcinoma, which presently is most amenable for concentrated experimental screening.

Mammary Tumors

Thus far, the best work is that of Huggins *et al.*,[1-3] who studied the effect of removal of various endocrine organs and the influence of hormones and hormone analogues on the growth of a transplanted fibroadenoma in Sprague-Dawley rats. In general, growth was stimulated by ovarian and reduced by testicular hormones and adrenalectomy. The greatest inhibition was produced by hypophysectomy.[1]

These investigators also studied the effects of several androstane compounds. In gonadectomized females, testosterone (17-β-hydroxyandrost-4-en-3-one) was most inhibitory.[3] Dihydrotestosterone (17-β-hydroxyandrostan-3-one) was almost as effective, while 2,2′-17-α-trimethyldihydrotestosterone greatly stimulated growth of the tumor.[2] Estrogen and progesterone, combined, increased tumor growth in hypophysectomized hosts; the addition of either growth hormone or prolactin with ovarian hormones further enhanced such growth.[1] None of these pituitary or ovarian hormones were effective when given alone.

The mammary glands of hypophysectomized tumor-bearing rats did not react to the hormones in the same manner as the tumor; growth hormone caused development of ducts and alveoli of the breast, but did not stimulate the tumor. Progesterone given to intact hosts accelerated tumor growth. This is in contrast to the pioneer work of Noble and Collip,[13] in which estrogen-induced mammary tumors of the rat were inhibited by progesterone. Furthermore, all androstane compounds tested by Huggins *et al.* that inhibited tumor growth stimulated growth of the mammary gland. Some of these compounds also inhibited the ovaries. Huggins *et al.* suggest two modes of action of the androstane compounds: (1) depression of hormone production by the ovary and (2) blocking activity of the growth-promoting steroids on the tumor cell.[2]

The role of anterior pituitary hormones in mammary growth and tumorigenesis requires much further investigation. In our experiments,[14-17] native rodent hormones released from secretory pituitary tumors in rats and mice proved to be many times more effective than commercial heterologous pituitary hormones. Furthermore, the relationship between purified growth hormone and prolactin requires clarification. In our experiments,[17] commercial preparations, both prolactin and growth hormone, induced body growth and development of the mammary glands in hypophysectomized animals (albeit when given at somewhat higher dosages than those used by Huggins *et al.*[1]).

It appears that stromal and epithelial elements of mammary tumors have different sensitivities, and that some human breast cancers respond to estrogens, while others respond to androgens. The situation has not been well analyzed, but obviously the use of a single type of benign tumor is inadequate for screening.

Much is known from the excellent work of Lyons *et al.*[18] and Cowie and Folley[19] on hormones affecting the normal breast. The basic promoter is estrogen; adrenal corticoids are powerful cofactors. Noble and Collip[13] described rat tumors that were fully dependent on estrogen, and Reynolds[20] noted recently a remarkable responsiveness of a mouse tumor to adrenalectomy, as noted earlier by Huggins and Mainzer[3] with fibroadenoma of the rat. Phenolic estrogen can have inhibitory effects in large doses and stimulating effects in small doses.[6] The role of the mammosomatotrope, through which estrogen apparently exerts its effect on the mammary gland in originating and maintaining tumor growth, is unknown. Antagonistic substances against these three types of hormones (estrogens, corticoids, and mammosomatotropins) or against cells secreting them should be the prime target of screening.

Our contribution is the isolation of a pituitary cell type named "mammotrope" or, more correctly, "mammosomatotrope." Tumors of this cell type can be induced by estrogens or ionizing radiations (the latter are ineffective without estrogens). Morphologically, this cell is *the* (or *a*) type of acidophile, as shown in the electron micrographs of Marilyn G. Farquhar, of the University of California Medical Center, San Francisco, Calif. Hormones can influence the breast and breast tumors by direct or indirect action. Estrogens act primarily by way of mammotropes; the beneficial effects of hypophysectomy, we believe, are due to removal of this mammosomatotrope, although corticoids are potentiating and modifying forces. Thus, in the search for chemotherapeutic agents active against breast cancer, one should include a search for mammotrope antagonists. We believe the screening object should be an animal host carrying both a grafted mammotrope and one or more mammary tumors of varying grades of malignancy and responsiveness. On the basis of results with leukemias, one might expect that different tumor strains do not respond alike to different inhibitors. Therefore, until some knowledge is gained by trial and error, the use of a spectrum of breast tumors is recommended

Haddow[21] reported in 1935 that 86 per cent of a large number of carcinogenic compounds tested inhibited tumor growth, and that there was a statistically significant correlation between carcinogenecity and tumor inhibition. Some aromatic hydrocarbons with little carcinogenicity also possess the property of restraining tumor growth.[21] Mutagenic, hormonal (estrogenic), carcinogenic, and carcinolytic properties of diverse compounds tested do not go hand in hand. Therefore, it is conceivable that substances might exist that possess predominantly carcinolytic activity. Huggins and Pollice[22] found recently that 3-methylcholanthrene retarded transplanted mammary tumors of the rat, and that this was brought about by profound inhibition of pituitary activity. The action was markedly enhanced by simultaneous administration of androstan-17-β-ol-3-one. 3-Methylcholanthrene also caused some improvement in inoperable breast cancers of women.[23]

For routine screening for mammary tumor inhibitors we propose the following three refinements: (1) the use of mammotropic tumors; (2) several mammary tumors of varying grades of malignancy and responsiveness; and (3) the use of highly inbred strains of rats, such as our Wistar strain or those of W. F. Dunning. The Sprague-Dawley strain is far from being homozygous. Cronkite *et al.*[24] have studied the transplantability and dependency of breast tumors of several types occurring in this strain and have found many failures, apparently due to lack of homozygocity.

We share the opinion of Huggins that, at present, breast tumors of the rat are more suitable than mouse tumors for screening. These tumors are either autonomous at the start or rapidly become so (perhaps the virus is an interfering factor). However, for reasons of economy in space, personnel, and expense, efforts should be continued to develop hormone-responsive breast tumors in mice.

Other Systems

Space does not suffice for a comprehensive review of the many experimental neoplasms in endocrines and their target systems (TABLE 1). George Woolley and Vincent Hollander have been working with adrenal tumors. The adrenal cortex is a complex organ containing at least three types of cells; consequently, it is capable of giving rise to three types of tumors. Two are available: the gonadal hormone-secreting type developed by Woolley and Little[25] and the glucocorticoid-secreting type isolated in our laboratory,[26] which causes a Cushing-like syndrome.

Prostatic tumors of both glandular and sarcomatous types induced by carcinogens have been described,[5] but not explored from the standpoint of screening.

All three types of pituitary tumors developed in our laboratory are of potential value in the search for inhibitors of tumors of their target organs. Of these, the mammosomatotropes already have been mentioned. The thyrotropes are of potential value in the search for inhibitors of functional thyroid tumors.[27] Theoretical considerations led us to suppose that, for inoperable responsive thyroid tumors, thyrotrope inhibitors may be of equal, if not greater, value than I^{131}. Clinical experience reviewed recently by Crile[28] supports this theory. Destruction of functional thyroid tissue with I^{131} is a stimulant of thyrotropic hormone (TtH) and, with it, of growth of residual responsive thyroid tumor. Thyroid hormone (TH) inhibits thyrotropes. However, the dose of thyroxine is limited by its toxicity and, therefore, search should be made for analogues that inhibit TtH, but are less toxic.

Cushing's disease is a tumor of adrenotropes; adrenotropic pituitary tumors now available[12-27] are autonomous, but some are fairly responsive and could therefore serve for screening compounds of potential benefit in this dysfunction.

There are possibilities for *in vitro* screening of potential inhibitors of hormones and endocrine tumors, but these will always call for supplementation with *in vivo* techniques, because function and responsiveness of cells do not go hand in hand. In general, it is true that highly secretory tumors are highly responsive, but there are exceptions. For example, Reynolds[20] found a highly responsive thyrotropic tumor with very low function, and we also have similarly behaving mammotropic tumors. Highly malignant adrenal tumor strains that metastasize extensively in the rat and the mouse proved to be highly responsive to AtH *in vitro*.[26] Sensitivity of one of these tumors equals that of the normal adrenal, so that it can serve for *in vitro* assays of AtH. The use of *in vitro* systems such as this, and that of Villee[29] for estrogens, are certainly worth while from the standpoint of detection of specific inhibitors of function. The Cancer Chemotherapy National Service Center, Bethesda, Md., already supports inhibition assays of androgenicity, using testosterone as the reference standard and the ventral prostate and seminal vesicles as targets. It also supports assays for antithymolytic activity. As said before, function and growth need not go hand in hand, and response of neoplastic cells can be abnormal, although usually it is in the same direction as that of the homologous normal cell. In spontaneous tumors, Foulds[30] found no apparent linkage between response by secretion and dependence of growth upon hormones. This limits the potential value of *in vitro* screening for growth inhibitors.

The potential value of tissue culture for screening for endocrine tumor inhibition and, incidentally, for hormone production, is excellent. Short-term growth *in vitro* in a highly functional state already has been recorded by us.[31] Growth and function of endocrines depends on the checks and balances of feedback mechanism. Theoretically, use of known specific promotors and inhibitors should make possible cultivation of endocrine cells *in vitro* with production of hormones. The monomorphous functional pituitary tumors are excellent material for such exploration.

On theoretical grounds, tumors of endocrines and their target organs are the most promising objects for chemotherapy. This is a task for many laboratories. The Endocrine Panel of the Cancer Chemotherapy National Service Center is available for consultation and assistance.

Conclusion

Experimental screening for growth antagonists of tumors related to endocrine organs, making use of their specific affinities, is an almost unexplored area of research that is of great promise for control of similar human neoplasms. Such research, supplemented with investigations of the hormonal states of man and animals in which such tumors are likely to originate, should attempt corrections of deviation in hormonal states predisposing neoplasia.

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Discussion of the Paper

Philip C. Merker* (*Division of Human Tumor Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, and Sloan-Kettering Division, Cornell University Medical College, New York, N. Y.*): Furth has presented interesting and new experimental tumor systems that should be explored and analyzed as to their usefulness in a chemotherapy program. However, a precautionary note should be added: these hormonally dependent systems should be used to study and understand cancer; they should not be lost in a maze of endocrinological exercises. The pituitary-adrenal-thyroid-uterus-breast-testis relationship has been well established experimentally. The fact that a hormonal relationship may exist for some types of human cancer is also well documented. Therefore, it does not suffice merely to redescribe these relationships with new procedures; these new tumor systems should be used in the light of experimental cancer chemotherapy. A case in point is the concept of dose: for the endocrinologist, amounts of agent are measured in terms of physiological dose levels; for the cancer chemotherapist, maximum tolerated doses can be used to determine antitumor effects, even though they may be many times greater than pre-established physiological dose levels.

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The literature on the chemotherapeutic uses of steroids contains many reports of effects against nonhormonally dependent animal tumors over a wide range of dosage and treatment schedules. Clinical reports also indicate that various steroids, androgens and estrogens in particular, sometimes produce objective improvement in osseous disease and soft part metastasis in advanced mammary carcinoma. As a result of these reports. there is keen interest in the development of other steroids that would have "carcinolytic action without undesirable side effects."[1] To understand better the experimental chemotherapy results, a portion of the literature concerned with testosterone was chosen for review.

Approximately 50 reports were examined, not only for the effect produced by testosterone on the tumor, but also for the route of administration used, timing and sequence of dosage, and the form of the drug—all important variables.[2, 3] In 30 of these reports the mouse was used as host, in 15 the rat, in 3 the rabbit, in 1 the guinea pig, and in 1 the hamster. In the mouse-tumor systems, transplantable tumors were employed in 33 per cent of the cases, spontaneous tumors in 43 per cent, and induced tumors in 21 per cent. In the rat-tumor systems, transplantable tumors were used in 69 per cent of the cases, spontaneous neoplasms in 5 per cent, and induced tumors in 26 per cent.

The analyzed reports reveal the following with respect to the effect on tumor growth: approximately 50 per cent considered testosterone negative in the particular tumor system; approximately 40 per cent classified the drug as "positive" or "active"; and 10 per cent classified the drug as having slight or questionable activity.

The literature that classified the drug as "negative" indicates that 17 of these effects were obtained against transplantable tumors, 8 were observed against spontaneous neoplasms, and the remaining 5 negative effects of the total 30 against induced tumors. A similar analysis was made of the tests that classified the drug as active or positive. The classifications can be separated as follows: 6 were obtained against transplantable tumors, 8 were observed against spontaneous neoplasms, and the remaining 10 positive effects of the total 24 were obtained against induced tumors. For convenience and brevity, only positive literature reports have been included in this report (TABLE 1).

Inspection of the literature reveals the different techniques used for the

TABLE 1

REPORTS IN THE LITERATURE INDICATING THAT TESTOSTERONE AND/OR ITS ESTERS HAVE ANTITUMOR ACTIVITY

Type of Tumor	Host	Substance	Dose (mg.)	Route	Solvent	Length of treatment	Observer
Spontaneous							
Mam.	Mice	Test.		S.C.	Pell t		Flaks[4]
Fibroma	Rat	Test. p.	5–15			Divided	Heiman[5]
Mam. ca.	Mice	Test. p.	8.7–18	Inj.		8–16 mo.	Heiman[6]
Mam. adeno.	Mice	Test. p. + progest.	0.2–6.0	S.C.	Oil	100+ doses	Heiman[7]
			0.2–0.6	S.C.	Oil	100+ doses	
Mam.	Mice	Test. p.	0.5/inj.	S.C.	Oil	41 weeks	Jones[8]
Mam. adenoca.	Mice	Test.	2.5		Oil	100+ days	Lacassagne & Raynaud[9]
Leuk.	Mice	Test. p.	3.0	S.C.	Pellet	50 weeks	Murphy[10]
Mam.	Mice	Test. p.	0.5			3 × weekly, 4 mo.	Nathanson & Andervont[11]
Transplanted							
Adenoca.	Mice	Methyl test.	100	Oral	Oil	13 days	Foley[12]
Adenofibr.	Rat	Test. p.	5–15			Divided	Heiman[5]
Mam. adenofibr.	Rat	Test. p. after progest. and estrogen	15:10			8 weeks	Heiman[13]
Adenofibr.	Rat	Test.	1.0/day			106 days	Millar & Noble[14]
Lymphosc.	Rat—pyridoxine deficient	Methyl test.	2.0	S.C.	Saline		Stoerk[15]

Induced by							
Chemicals:							
Mam.	Rat ♀	Test. p.	0.5	I.M.	Oil	3 × weekly, 92–400 days	Cantarow *et al.*[16]
Skin	Rat	Test. p.	62.5–75.0	S.C.	Oil or pellets	10–12 weeks	Flaks[4]
Prostatic	Mice	Test p.			Pellet		Horning[17]
Estrogen:							
Mam. adenoca.	Mice	Test.	2.5		Oil	100+ days	Lacassagne & Raynaud[9]
Lymphoid	Mice	Test. p.	0.625; 1.25; 2.5	S.C.	Oil	10 weeks to life span	Gardner *et al.*[18]
Kidney	Hamster	Test. p.	2.5–20.0		Oil	10 weeks	Horning[19]
Fibroid	Guinea pig	Test. p.	0.130				Lipschutz[20]
Castration:							
Adrenal adenoma	Mice	Test. p.	0.250			Weekly	Monsen[21]
Adrenal adenoma	Mice	Test. p. Test. dip.		S.C.	Pellets	From 2 mo. after birth	Woolley & Chute[22]
X Ray:							
Lymphoid	Mice	Test.-AlPO$_4$ gel (25 mg./cc.)	0.02		Al phosph. gel	2 × weekly, 10 weeks	Kaplan & Brown[23]

Legend: mam. = mammary; test. = testosterone; test. p. = testosterone propionate; progest. = progesterone; test. dip. = testosterone dipropionate.

actual test procedures. The drug was administered subcutaneously (by injection or pellet implantation), intramuscularly, and orally; oil, saline, or other media were used to dissolve or suspend the drug prior to its use. The length of treatment varied from 1 week to the life span of the animal, and the doses ranged from 0.2 to 15 mg. per injection to long-term pellet depot doses. There was no uniformity for species, strain, sex, or age of host.

One would presuppose that any experimental animal tumor or testing regime that failed to detect anticancer activity in testosterone would be subject to suspicion as a useful means of detecting other anticancer substances of this class. If all of the systems that failed are discarded, a sufficiently large spectrum of tumor types and regimes remain to satisfy most investigators.

If a "large-scale preliminary screening" program is to be considered, there is much to be desired in some of the tumor-host systems that have classified testosterone as positive. For one, those that required more than 400 days for a test or complicated techniques for "inducing" tumors would be of questionable value for screening many compounds in a short period of time. An important contribution could be made to the experimental chemotherapy field if some of the long-term tumor host systems were restudied with the intent of shortening the period required for an adequate test. However, long-term systems offer the opportunity to test steroids for anticancer activity over an extended period of treatment and, at the same time, to supply important chronic toxicological and endocrinological data. This dual service to experimental cancer research should not be overlooked.

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R. L. NOBLE (*University of Western Ontario, London, Ont., Canada*): I think one might inquire as to what truly hormone-dependent tumors are available. Merker referred to studies with testosterone, but most of the tumors used were not hormone-dependent in the endocrine sense. We might therefore use a hormone such as testosterone simply as a chemotherapeutic agent and screen it against the list of ordinary tumors. In such a screening procedure the hormone may react directly on the tumor and thereby appear as active. On the other hand, hormones or synthetic compounds may have more specialized properties that might be apparent if one were to use a hormone-dependent type of tumor system. It is important to differentiate properly between these two types of screening procedures.

The tumors that show hormonal dependency and that are available at the moment are: (1) the guinea pig uterus benign tumor described by Lipschutz, which is dependent on estrogens and can be antagonized by progesterone, deoxycorticosterone, or testosterone; (2) a carcinoma that grows in the flank organ of the hamster, induced by testosterone and dependent on it for growth; and (3) the well-known interstitial testicular tumors in the mouse, which may be dependent on estrogens. Also, one has the kidney tumor in the male hamster, which is entirely estrogen-dependent. There has been some work on dependent carcinomas of the prostate, which were induced by carcinogens, as opposed to the induced squamous cell cancer of the prostate, which is not hormone-dependent. Apparently the only androgen-dependent tumors are the occasional cancers of the prostate in the rat or the mouse and in the organ in the flank of the hamster.

The question of how these tumors would differ from ordinary tumors, if used for screening procedures, is a difficult one. Hormone-dependent tumors possess the curious property of progression so well described by L. Foulds. By progression I mean a change in the characteristics and possible responsiveness of the tumor. In dealing with a transplanted hormone-dependent tumor, therefore, the response to different hormones may change as the generations proceed. This raises a difficult problem, then, as to the stage of progression the tumor has achieved at any given time.

Unfortunately, since the hormone-dependent tumors are either spontaneous or induced, the time intervals are so long that they are really not practicable for chemotherapeutic studies. Ideally, therefore, a transplantable hormone-dependent tumor in which the transplants will retain indefinitely their hormonal dependency is desirable.

Some studies on rats with two different types of dependent mammary tumors have been reported. I shall illustrate some of the special features of these tumors.

The uniqueness of the hormone-dependent mammary tumor might be attributed to the fact that we have a comparable dependent tumor in the human; the earliest studies in this field actually originated in the clinic in breast and prostatic cancer. A fibroadenoma used by Huggins' group is simply an enormous benign tumor that grows slowly and progressively. Most of us would say this tumor should not even be considered for screening purposes. Curiously enough, as shown by Huggins and others, this tumor does respond to hormones, does require estrogen for growth, and can be antagonized growthwise by, for instance, removal of the ovaries or transplantation into male animals. In the experience of my associates and myself many tumors of this type show progression, so that after four or five generations they can be transplanted equally well into males and females, whereas initially they would grow only in the female. We feel that this demonstration illustrates a change in the characteristics and response of these tumors that is a danger when using them for screening. Earlier generations would have seemed estrogen-dependent; inhibition of estrogen would cause inhibition of the tumor. The tumor of later generations developed the capacity to grow without estrogen, which would be misleading to the investigator.

A different type of hormone-dependent mammary tumor can be induced in the rat by subcutaneous pellets of estrogen. These multiple tumors, in contrast to the preceding essentially histologically benign tumors, appear histologically as typical adenocarcinomas. Although these carcinomas may infiltrate and occasionally metastasize, they are dependent entirely on the pellet of estrogen that has induced them, and they disappear quite dramatically with its removal. A short time after the pellet is removed, the tumors begin to shrink and, within a week or two, they have essentially disappeared. This has been a consistent finding in our own experiments. Curiously enough, we have not been able to transplant these tumors even into rats of the same strain bearing estrogen pellets. On the other hand, if the pellet is replaced after the primary tumor has regressed, there is a rapid regrowth of the tumor. By this means it is possible to shorten the time in which these tumors can be induced.

I believe hormone-dependent tumors in the rat raise other points of interest. Unfortunately, their counterpart in the human behaves differently. Nowadays, the surgeon, after removing the breast, removes the ovaries and then the adrenals and, eventually, even the pituitary gland. Although many cases of breast tumors in humans go through stages of hormone dependency and may be checked by these depletion operations, eventually there is always a form that is hormone-independent. If we could duplicate in the human what has been shown in the rat, where the tumors remain hormone-dependent, I think we might anticipate some cures of human cancer of the breast. The mechanism of progression of changes in characteristics of these tumors is an important and suitable field for study, and might perhaps lend itself to observations on the effects of chemotherapeutic agents.

FURTH: The changes occurring in transplantable tumors are no longer too disturbing, since the tumors can be preserved in a frozen state and resuscitated when wanted years later. Furthermore, strains vary with respect to likelihood of changes in the course of transplantation. The major change occurs when a spontaneous tumor is first transplanted into normal animals. Thereafter, with respect to secretory capacity and responsiveness, many strains remain fairly constant over long periods of time. Primary screening should be done with such fixed lines, and final screening with primary tumors.

RESPONSE OF SOME ISOLOGOUSLY TRANSPLANTED RAT NEOPLASMS TO STEROIDS*

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In view of the difficulty experienced in obtaining suitable dependent or hormone-responsive neoplasms for evaluating the effectiveness of hormone therapy, and the relatively long and variable latent period and low growth potential of those that have been observed, it seemed worth while to explore the possibility of evaluating the effectiveness of steroid therapy on some of the more rapidly fatal isologously transplantable rat neoplasms of diverse morphology.

For this purpose the following doses of steroids were injected subcutaneously for as long as tolerated in rats inoculated with seven different neoplasms. The chemicals tested thus far include: cortisol (10 or 20 mg./kg.); testosterone (10 or 20 mg./kg.); estrone (50 or 100 μg./kg.); deoxycorticosterone (30 mg./kg.); and progesterone (5 or 50 mg./kg.). All were suspended in SPF 17874 of the Upjohn Company of Kalamazoo, Mich., and injected daily in 0.2 ml. doses in the subcutaneous tissues at a distance from the site of inoculation of the neoplasm. The controls were similarly injected with 0.2 ml. of the suspending fluid.

The 7 neoplasms were each treated with all 5 of the steriod compounds as follows:

(1) R92, a spontaneous sarcoma of the sternum, described in 1922 by Bullock and Curtis[1] and transplanted for 422 transfer generations in rats of Zimmerman line 61.

(2) R2788, a spontaneous lymphosarcoma of the illeocolic mesentery,[2] observed in 1947 in a piebald rat of A × C line 9935 from the thirtieth brother-by-sister generation. This tumor has been carried for 138 transfer generations in rats of this line.

(3) R2426, a spontaneous adenocarcinoma of the mammary gland[3] that arose in a rat of the twenty-seventh brother-by-sister generation of August line 7322 and is currently in the one hundred and fifteenth transfer generation in rats of this line.

(4) and (5) M-C961 and M-C972, both squamous cell carcinoma of the prostate induced by methylcholanthrene as described in 1946.[4] These tumors were observed in 1956, respectively, in rats of the forty-second and thirty-ninth brother-by-sister generations of A × C line 9935 Irish rats and are currently in the thirty-third and seventeenth transfer generations.

(6) L-C18, a malignant hepatoma[5] induced by acetylaminofluorene in a Fischer line 344 rat of the forty-second brother-by-sister generation of the line. The tumor is now in the eighty-eighth transfer generation.

(7) IRC741, an acute leukemia[6] that occurred spontaneously in a Fischer line 344 rat of the fifty-first brother-by-sister generation of the line. This tumor is currently in the one hundred and thirty-fifth transfer generation.

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TABLE 1

ACUTE LEUKEMIA IRC741/128A

Compound	Daily dose	No. inj.	Number		Initial body wt.	Days to death (mean ± P.E.)	Tumor diam. at 13 days (mean ± P.E.)	Body wt.	WBC at 15 days
			Rats	Tumors					
SPF 17874	0.2 cc.	13	9	9	182	16 ± 0.15	3.2 ± 0.06	190	148,000
Cortisol	4–2 mg.	14	10	10	185	19 ± 0.34	1.7 ± 0.12	151	21,300
Estrone	10 μg.	13	10	10	173	16 ± 0.14	3.0 ± 0.08	167	138,800
D.O.C.	6 mg.	13	10	10	182	16 ± 0.14	3.4 ± 0.04	185	98,500
Progesterone	1 mg.	13	10	10	176	16 ± 0.21	3.2 ± 0.06	179	144,300
Testosterone	2 mg.	15	10	10	171	18 ± 0.14	3.1 ± 0.05	180	63,000

All of these neoplasms except L-C18 grow progressively in 100 per cent of the inoculated rats of the line of origin. The exception, L-C18,[7] has regressed spontaneously in about 10 per cent of the inoculated rats of some series, but no regressions have been observed in the current series.

The tumors were inoculated subcutaneously on the right side or bilaterally as 2- to 3-mg. grafts of tumor tissue in adult intact males that weighed approximately 200 gm. Groups of 10 were assigned randomly as controls, or to the

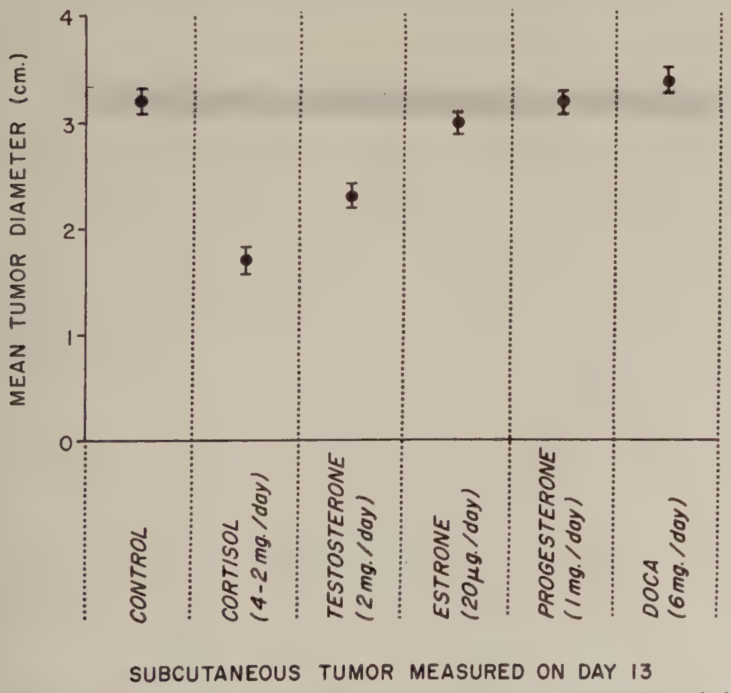

FIGURE 1. Effect of hormonal agents on leukemia IRC741: a comparison of the mean tumor diameter of each treated group with the mean ± P.E. of the control group.

chemical to be tested, and the injection schedule was initiated 24 hours after inoculation. The rats were weighed and inspected for tumors weekly thereafter. As soon as they were palpable, the tumors were measured. In each case the three largest diameters were recorded in centimeters, and an average diameter was obtained for each tumor. The injection schedule was maintained until the death of any one of the group appeared imminent. Following the completion of the injection schedule, the rats were observed for growth of the neoplasms, survival data, and post-mortem examination.

Table 2
Lymphosarcoma R2788/131A

Compound	Daily dose	No. inj.	Number		Initial body wt.	Days to death (mean ± P.E.)	18 Days	
			Rats	Tumors			Tumor diam. (mean ± P.E.)	Body wt.
SPF 17874	0.2 cc.	22	10	20	237	27 ± 0.51	3.2 ± 0.07	247
Cortisol	4–2 mg.	17	10	20	214	28 ± 0.43	2.3 ± 0.10	198
Estrone	10 μg.	19	10	20	219	24 ± 0.49	3.6 ± 0.10	235
D.O.C.	6 mg.	19	9	18	228	25 ± 0.76	3.6 ± 0.08	258
Progesterone	1 mg.	22	10	20	215	26 ± 0.28	3.2 ± 0.08	230
Testosterone	2 mg.	21	10	20	214	26 ± 0.98	3.1 ± 0.18	237

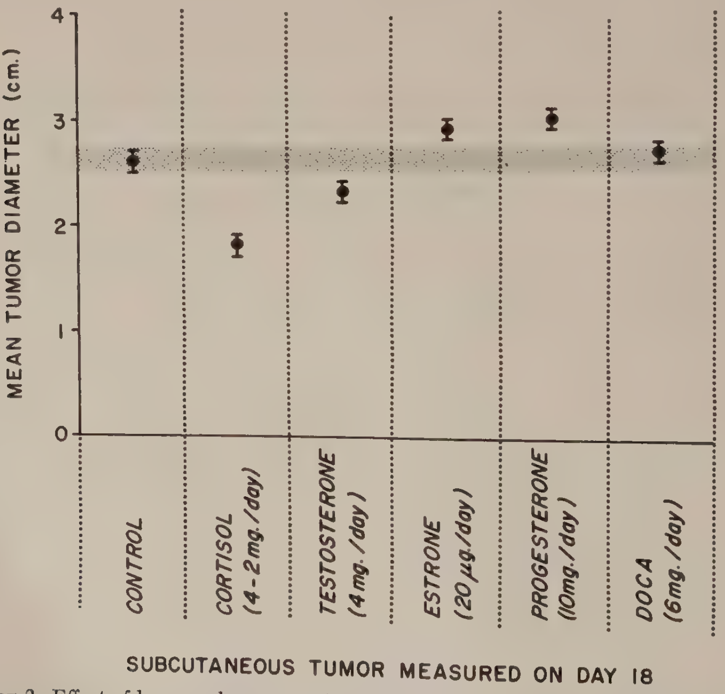

Figure 2. Effect of hormonal agents on lymphosarcoma R2788: a comparison of the mean tumor diameter of each treated group with the mean ± P.E. of the control group.

Tables 1 to 7 and figures 1 to 7 summarize the data obtained for the different neoplasms. Table 1 shows that rats inoculated with acute leukemia IRC741/128A survived longer than the controls or other treated groups when they were treated with cortisol and testosterone. In figure 1 the average diameters of the tumors in the control group plus and minus the probable error is compared with the average diameters of the tumors in each treated group. The tumors were significantly smaller in the cortisol-treated rats. At 15 days after inoculation (table 1), the average white blood cell count

Table 3

Sarcoma R92/414A

Compound	Daily dose	No. inj.	Number		Initial body wt.	Days to death (mean ± P.E.)	19 Days	
			Rats	Tumors			Tumor diam. (mean ± P.E.)	Body wt.
SPF 17874	0.2 cc.	24	10	10	252	32 ± 0.77	3.4 ± 0.09	257
Cortisol	4–2 mg.	12	10	10	228	32 ± 1.9	2.8 ± 0.21	192
Estrone	10 μg.	25	10	10	236	35 ± 0.90	3.0 ± 0.10	224
D.O.C.	6 mg.	24	10	10	217	32 ± 2.2	3.2 ± 0.05	216
Progesterone	1 mg.	25	10	10	221	35 ± 1.1	3.1 ± 0.16	221
Testosterone	2 mg.	24	10	10	230	31 ± 0.50	3.3 ± 0.10	236

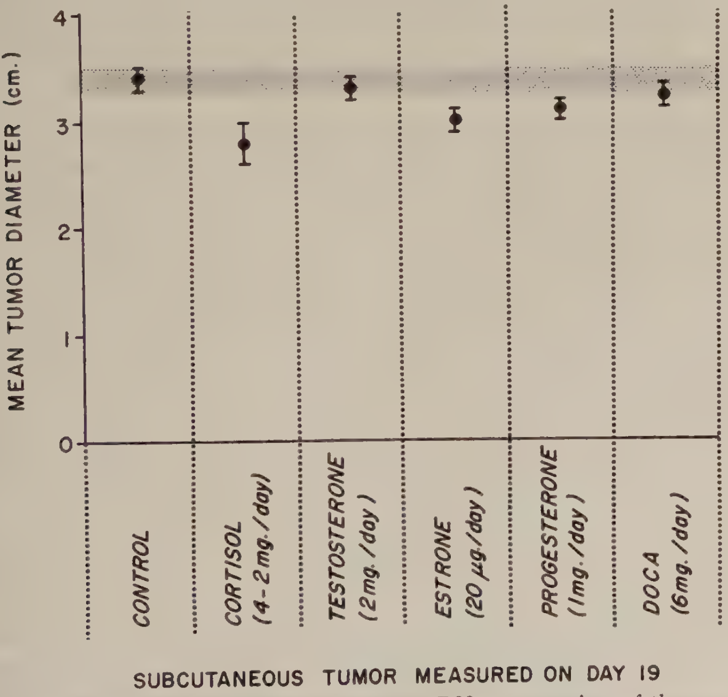

Figure 3. Effect of hormonal agents on sarcoma R92: a comparison of the mean tumor diameter of each treated group with the mean ± P.E. of the control group.

TABLE 4

SQUAMOUS CELL CARCINOMA M-C961/27A OF PROSTATE

Compound	Daily dose	No. inj.	Number		Initial body wt.	Days to death (mean ± P.E.)	24 Days	
			Rats	Tumors			Tumor diam. (mean ± P.E.)	Body wt.
SPF 17874	0.2 cc.	26	8	16	218	36 ± 1.0	2.6 ± 0.11	217
Cortisol	4–2 mg.	7	10	20	215	44 ± 4.5	2.9 ± 0.05	190
Estrone	10 μg.	26	10	20	209	42 ± 2.0	2.6 ± 0.06	197
D.O.C.	6 mg.	26	9	18	217	38 ± 2.0	2.7 ± 0.08	215
Progesterone	1 mg.	26	10	20	218	49 ± 3.4	2.7 ± 0.06	214
Testosterone	2 mg.	26	10	20	214	50 ± 4.7	2.5 ± 0.08	219

TABLE 5

SQUAMOUS CELL CARCINOMA M-C972/12A OF PROSTATE

Compound	Daily dose	No. inj.	Number		Initial body wt.	Days to death (mean ± P.E.)	24 Days	
			Rats	Tumors			Tumor diam. (mean ± P.E.)	Body wt.
SPF 17874	0.2 cc.	33	10	20	237	54 ± 1.7	2.1 ± 0.05	234
Cortisol	4–2 mg.	7	8	16	215	45 ± 1.8	2.4 ± 0.06	204
Estrone	10 μg.	31	10	20	205	48 ± 1.6	2.2 ± 0.08	194
D.O.C.	6 mg.	30	9	18	192	45 ± 1.8	2.6 ± 0.13	212
Progesterone	1 mg.	33	10	20	206	54 ± 2.5	2.5 ± 0.08	212
Testosterone	2 mg.	33	10	20	215	48 ± 1.5	2.3 ± 0.04	220

(WBC) was only 21,300 cells/cu. mm. for rats treated with cortisol, as compared with 148,000 for the untreated controls. The average WBC of the testosterone-treated rats (63,000) was leukemic at this time, but also was significantly lower than for the controls. Thirteen days after inoculation, the cortisol-treated rats had lost an average of 18 per cent in body weight, as compared with moderate gains in body weight for most of the other groups.

TABLE 2 and FIGURE 2 show a significant decrease in the average diameter of lymphosarcoma R2788/131A at 18 days after inoculation in cortisol-treated rats that lost less than 10 per cent in body weight. The growth of this neoplasm appeared to be stimulated slightly by treatment with estrone and deoxycorticosterone. This is shown by an increase in average diameter from 3.2 ± 0.07 cm. for the tumors in controls to 3.6 ± 0.10 in the treated rats, and a slight reduction in average survival for both treated groups.

TABLE 3 shows no significant differences in the growth of R92/414A or the average survival of the inoculated rats as a result of treatment with any of the compounds although, as shown in FIGURE 3, the tumors were slightly smaller at 19 days in the cortisol-treated rats.

TABLES 4 and 5 and FIGURES 4 and 5 show that the average diameters of the transplanted squamous cell carcinomas of the prostate M-C961/27A and M-C972/12A were larger at 24 days after inoculation in the cortisol-, deoxy-

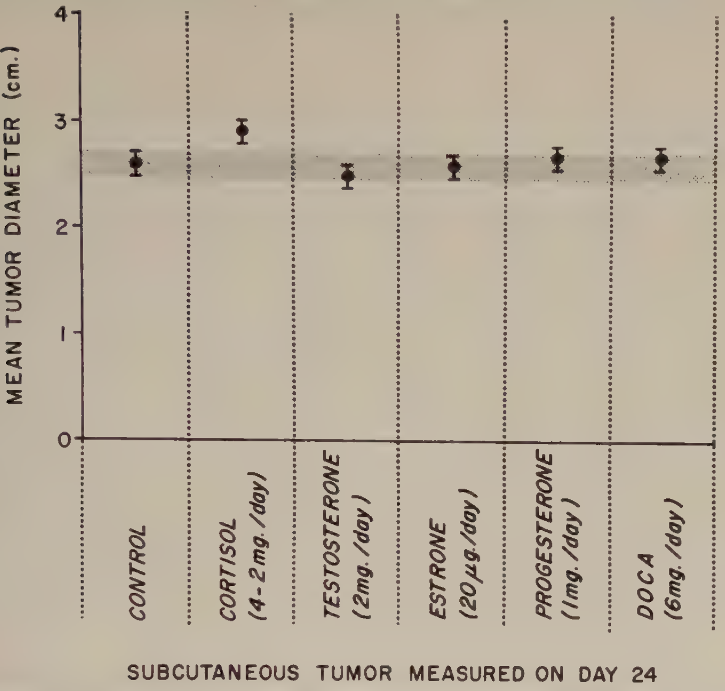

FIGURE 4. Effect of hormonal agents on squamous cell carcinoma M-C961: a comparison of the mean tumor diameter of each treated group with the mean ± P.E. of the control group.

corticosterone-, and progesterone-treated rats than in the controls or the estrone- and testosterone-treated rats.

The results of treatment of rats bearing the transplanted adenocarcinoma R2426/113A of the mammary gland are shown in TABLE 6 and FIGURE 6. The average diameters of the tumors at 31 days after inoculation were significantly smaller after cortisol and testosterone treatment in rats that gained in body weight than were the tumors of the controls or the other treated groups. On the other hand, deoxycorticosterone appeared to stimulate the growth of this tumor.

TABLE 7 and FIGURE 7 show for the same compounds the average diameter at 18 days after inoculation of the malignant hepatoma L-C18/87A. For this series the dose of progesterone was increased 10 times to 10 mg.; for testosterone and estrone the daily dose was doubled, increasing it, respectively, to 4 mg. and 20 µg. Except for two groups, those treated with cortisol and deoxycorticosterone, the rats were lighter in weight at the start of the experiment than were the rats of the previous series. In all groups of this series the treatment was suspended at 18 days after inoculation, when the tumors were first measured. The data show that cortisol significantly reduced the diameter of the tumors in rats that lost an average of 12 per cent in body weight, while testosterone significantly reduced the growth of the tumors in rats that

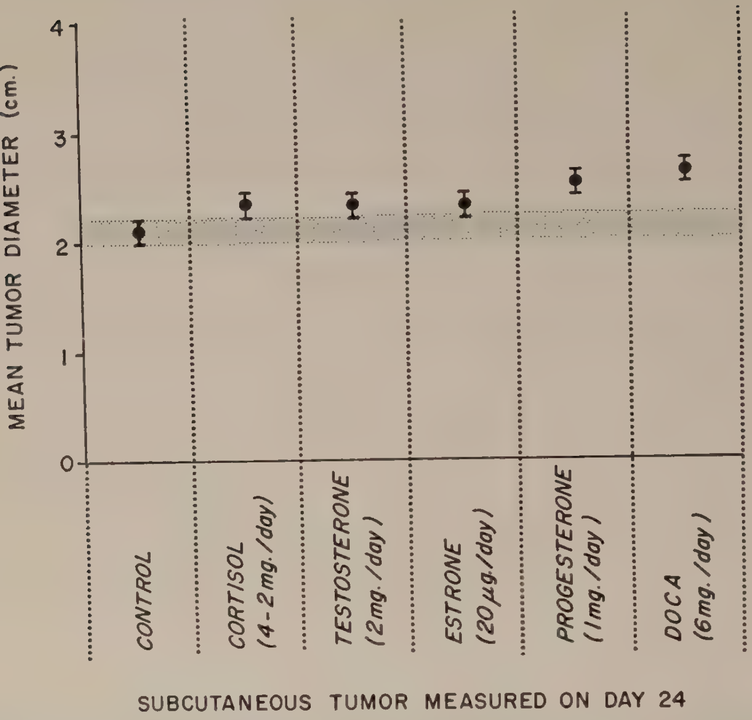

FIGURE 5. Effect of hormonal agents on squamous cell carcinoma M-C972: a comparison of the mean tumor diameter of each treated group with the mean ± P.E. of the control group.

TABLE 6

ADENOCARCINOMA R2426/113A OF MAMMARY GLAND

Compound	Daily dose	No. inj.	Number		Initial body wt.	Days to death (mean ± P.E.)	31 Days	
			Rats	Tumors			Tumor diam. (mean ± P.E.)	Body wt.
SPF 17874..........	0.2 cc.	57	11	22	196	95 ± 4.1	1.1 ± 0.04	207
Cortisol...........	4–2 mg.	21	9	18	171	105 ± 3.4	0.8 ± 0.07	177
Estrone...........	10 µg.	43	10	20	176	81 ± 6.0	1.2 ± 0.04	179
D.O.C.............	6 mg.	51	9	18	156	91 ± 4.3	1.4 ± 0.06	170
Progesterone.......	1 mg.	56	10	20	182	98 ± 4.9	1.1 ± 0.04	188
Testosterone.......	2 mg.	57	9	18	183	96 ± 3.4	0.8 ± 0.04	207

gained in body weight. Both estrone and progesterone stimulated the growth of this tumor.

The standard deviations of the average diameters of the tumors of this group are included in TABLE 7 to show the uniformity that may be obtained in the measurement of these tumors at a specified interval after inoculation. The average survival period for the rats of this series is not yet available.

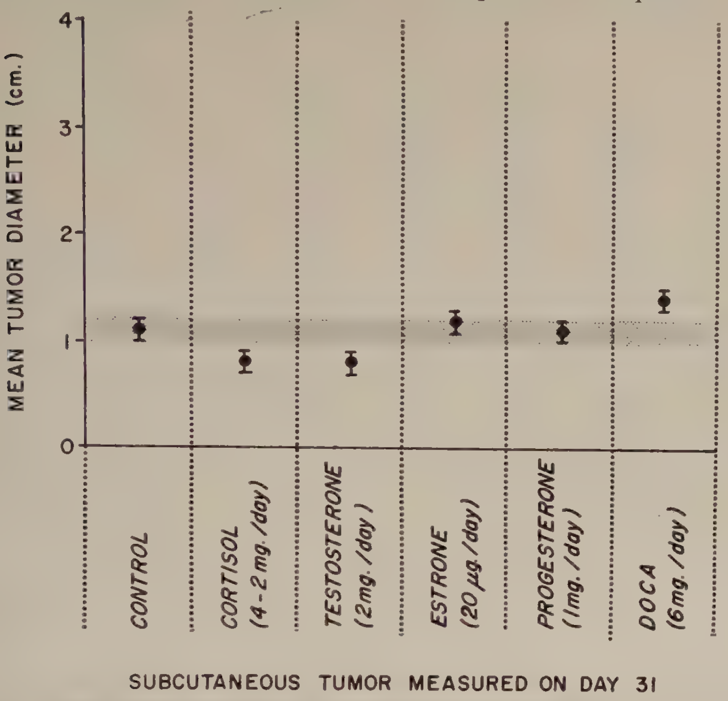

FIGURE 6. Effect of hormonal agents on mammary gland adenocarcinoma R2426: a comparison of the mean tumor diameter of each treated group with the mean ± P.E. of the control group.

TABLE 7
MALIGNANT HEPATOMA L-C18/87A

Compound	Daily dose	No. inj.	Number		Initial body wt.	18 Days		
			Rats	Tumors		Tumor diam. (mean ± P.E.)	Tumor diam. (S. D. ± P.E.)	Body wt.
SPF 17874	0.2 cc.	18	10	20	168	2.6 ± 0.06	0.4 ± 0.04	177
Cortisol	4–2 mg.	11	8	16	204	1.7 ± 0.08	0.9 ± 0.10	179
Estrone	20 µg.	18	10	20	123	2.9 ± 0.06	0.4 ± 0.05	131
D.O.C.	6 mg.	18	10	20	191	2.6 ± 0.07	0.5 ± 0.05	194
Progesterone	10 mg.	18	10	20	149	3.0 ± 0.05	0.4 ± 0.04	162
Testosterone	4 mg.	18	10	20	116	2.3 ± 0.07	0.5 ± 0.05	146

Although the differences in average diameter of the tumors are relatively small, they appear to be sufficiently dependable to be used in evaluating the effectiveness of carcinostatic activity. A standard procedure for each of the neoplasms might be adopted that could be evaluated between the tenth and thirtieth days after inoculation, depending upon the growth habit of the selected

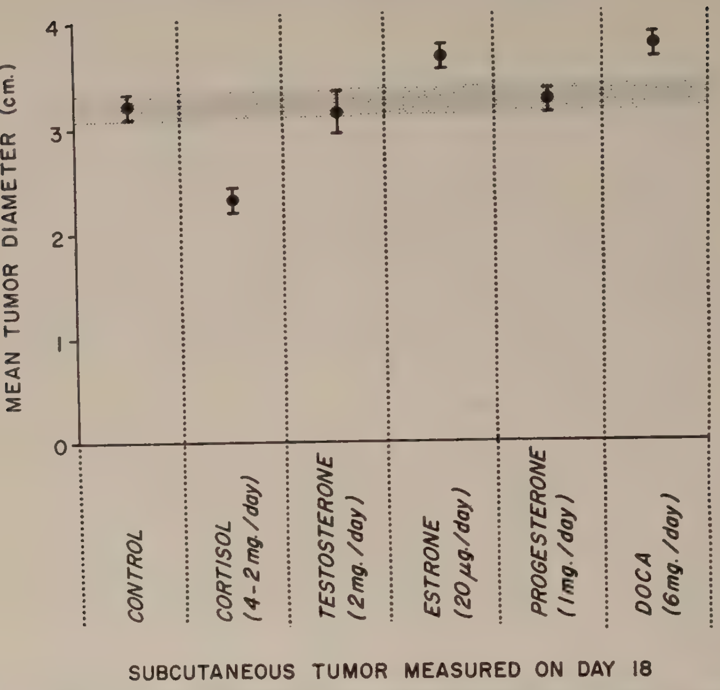

FIGURE 7. Effect of hormonal agents on the malignant hepatoma L-C18: a comparison of the mean tumor diameter of each treated group with the mean ± P.E. of the control group.

neoplasm. Further exploration may reveal more favorable dosage schedules that might be demonstrated by significantly increased survival periods for rats treated with each of the steroid compounds; however, the current tests indicate that qualitatitive differences in the compounds may be detected with considerable economy of material and time by an evaluation of comparable differences in average tumor diameter at a specified interval after inoculation of isologously transplanted neoplasms of diverse morphology.

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ESTRADIOL-SENSITIVE ISOCITRIC DEHYDROGENASE IN MAMMARY TISSUE*

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Many modalities of hormonal therapy are known to be of benefit to the patient with advanced breast cancer. Pearson and his associates[1] have shown that approximately half the premenopausal patients treated by oophorectomy may expect temporary remission of advanced mammary cancer. This remission was shown by metabolic and clinical studies to be due to the reduction in estrogen production accompanying the removal of the ovaries. These studies suggested that human breast cancer was of two types. The first is an estrogen-sensitive type that grows more slowly when major sources of estrogen are removed from the body and grows more rapidly when estrogenic hormones are administered. The second is an estrogen-insensitive type of tumor. When premenopausal women who had achieved benefit from oophorectomy relapsed and were treated subsequently by means of bilateral adrenalectomy, there was objective improvement in approximately half of the patients so treated. When bilateral adrenalectomy was performed on premenopausal patients who failed to benefit from oophorectomy, no striking remissions were obtained. These observations suggest that adrenalectomy also brings about improvement in patients with mammary cancer by removing a substantial source of estrogen from the body. Morphologic studies have failed to permit separation into estrogen-sensitive and -insensitive mammary cancer, although there is a tendency for the more differentiated tumor to be hormonally responsive.[2-4] An *in vitro* procedure that could predict the *in vivo* response of a breast tumor would have obvious clinical utility.

The present report concerns the separation of human breast cancer into two types, one that is sensitive *in vitro* to estradiol-17β and one that is not.

Estrogen-Sensitive Isocitric Dehydrogenase-DPN in Mammary Tissue

Villee[5-7] has described an isocitric dehydrogenase-DPN (diphosphopyridine nucleotide) in placental homogenates that is sensitive to estradiol-17β. This enzymatic system is also present in endometrium[8] and mammary tissue.[9-11] These tissues are target organs for the action of estrogenic hormone. The enzymatic system has not been found in organs that are not sensitive *in vivo* to estrogen. Recently the estrogen-sensitive system in placenta has been shown to consist of isocitric dehydrogenase-TPN (triphosphopyridine nucleotide) coupled with a steroid transhydrogenase.[12]

Nature of the Enzyme

The reaction in mammary homogenates stimulated by estradiol-17β has been shown[10] to be d-isocitrate + DPN $\rightarrow$ α-ketoglutarate + DPNH (reduced diphosphopyridine nucleotide).

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TABLE 1

COENZYME ACTIVITY OF STEROIDS FOR ISOCITRIC DEHYDROGENASE

Compound	Concentration (μg./ml. reaction mixture)		
	0.1	1.0	10.0
	per cent	*per cent*	*per cent*
Estradiol	100.0	100.0	100.0
3-Methoxyestradiol-17β	62.3	102.7	95.6
3-Methoxyestrone	35.0	85.7	92.6
3-Deoxyestradiol-17β	0.0	17.9	51.0
3-Deoxyequilenin	40.4	96.4	146.9
Equilenin	115.0	109.0	132.0
17-Deoxyestrone	0.0	0.0	0.0

The compounds were added at the indicated concentrations to a reaction mixture consisting of 0.30 ml. placental supernatant, 0.006 M phosphate buffer, 0.006 M $MgCl_2$, 0.001 M Versene, 0.002 M *dl*-isocitrate, 0.00035 M DPN in a final volume of 1.0 ml. Incubation was for 1 hour at 30° C. The enzymatic activity is expressed in terms of ketoglutarate formation relative to estradiol-17β.

The activity resides in the supernatant fraction of the homogenate after centrifuging 45 min. at 50,000 g. Estradiol potentiation of isocitric dehydrogenase activity has been detected by spectrophotometric measurement of DPNH, as well as by α-ketoglutarate formation. No cofactor other than a suitable divalent cation and DPN has been demonstrated. Co^{++}, Mg^{++}, or Mn^{++} serve as cation. An unknown heavy metal inhibitor is probably present in some breast homogenates, since 0.001 M Versene enhances the estradiol sensitivity of some specimens. The *p*H optimum in 0.006 M phosphate buffer is 6.8. The system shows an optimum substrate concentration of 0.0008 M *d*-isocitrate. Neither inhibition by, nor utilization of, *l*-isocitrate can be demonstrated. For this reason, synthetic racemic isocitrate is a convenient source of substrate. Estradiol-17β produces a maximum stimulation of the dehydrogenase at a concentration of 4×10^{-6} M. Recent studies by Villee[6] and in this laboratory[13] have shown that the 17β position of estradiol is of prime importance for steroid coenzyme activity in the placental estrogen-sensitive system. TABLE 1 shows that the phenolic hydroxyl is not essential for activity, and that there is little relationship between *in vivo* and *in vitro* estrogenic action, since the 3-methoxy or deoxy compounds tested are extremely weak estrogens *in vivo*. While equilin and equilenin exhibit detectable *in vitro* effects at 4×10^{-10} M, and while estradiol has a limit of detection of 4×10^{-8} M,[13] no advantage has been found for the former compounds in the assay of mammary homogenates.

Estradiol-Sensitive Enzyme in Mammary Cancer

FIGURE 1 shows typical spectrophotometric assays for estradiol sensitivity in a tumor homogenate stimulated by estradiol (1 μg./ml.), and in tumor homogenate not stimulated by estradiol. The spectrophotometric method employed[10] measures the rate of accumulation of DPNH in the presence of the 1 μg./ml. dosage of estradiol-17β.

TABLE 2 demonstrates typical assays for *in vitro* sensitivity of mammary cancers by measurement of the ketoglutarate production in the presence and absence of estradiol. Both sensitive and insensitive tumor homogenates are demonstrated. TABLE 3 summarizes our experience to date in separating breast cancer into estradiol-sensitive and -insensitive types. It is of interest

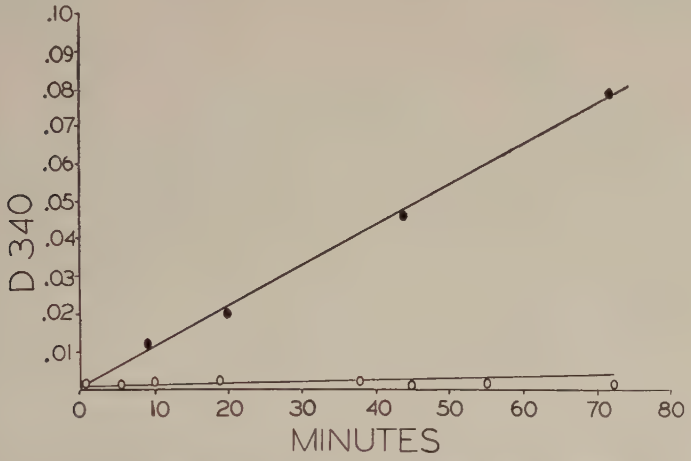

FIGURE 1. Typical spectrophotometric assay for *in vitro* estradiol sensitivity in homogenates of mammary cancer. Each assay system consists of two cuvettes containing 0.30 ml. homogenate, 0.10 M tris buffer *p*H 7.45, 0.002 M *dl*-Na_3 isocitrate, 0.003 M $CoCl_2$, 0.006 M nicotinamide, and 0.00025 M DPN in a total volume of 1.0 ml. The second cuvette contains, in addition, 1 μg./ml. estradiol-17β. The optical density difference between the cuvettes at 340 μM is plotted against time in minutes. Symbols: ● = homogenate exhibiting sensitivity; ○ = homogenate exhibiting no sensitivity.

TABLE 2

In Vitro ESTRADIOL-SENSITIVE AND -INSENSITIVE BREAST CANCER

Patient	Age	Microscopic diagnosis	μM KG/3 hr. without $E_2 \pm$ S.D.	μM KG/3 hr. with $E_2 \pm$ S. D.	μM KG/3 hr. due to E_2	P	N	Percentage increase
A. M. D.	38	Scirrhous Ca.	0.0130 ± 0.002	0.0229 ± 0.003	0.0099 ± 0.0019	<0.01	4	76.2
E. M. J.	48	Inf. duct Ca.	0.0456 ± 0.010	0.0707 ± 0.003	0.0251 ± 0.006	<0.02	3	55.0
M. S. B.	71	Inf. duct Ca.	0.034	0.107	0.073		1	214.7
D. B. O.	42	Inf. duct Ca.	0.0405 ± 0.003	0.0570 ± 0.004	0.0165 ± 0.0037	<0.05	2	39.5
J. C. R.	50	Inf. duct Ca.	0.0317 ± 0.0045	0.0447 ± 0.0022	0.0120 ± 0.0028	<0.02	3	36.7
R. E.			0.0428 ± 0.0038	0.0405 ± 0.013	−0.0023 ± 0.009	N.S.	3	—
H. B. S.	44	Inf. duct Ca.	0.0589 ± 0.001	0.0607 ± 0.001	0.0018 ± 0.0014	N.S.	3	—
A. M. S.	69	Inf. duct Ca.	0.0563 ± 0.0024	0.0539 ± 0.002	−0.0024 ± 0.0022	N.S.	3	—
J. Y. A.	64	Inf. duct Ca.	0.0151 ± 0.003	0.0146 ± 0.0037	−0.0004 ± 0.005	N.S.	2	—

The enzyme used was the supernatant fraction of tumor homogenate. The incubation mixture was 0.30 ml. homogenate, 0.006 M phosphate buffer *p*H 6.8, 0.006 M $MgCl_2$, 0.001 M Versene, 0.002 M *dl*-isocitrate, and 0.00035 M DPN in a total volume of 1.0 ml. Incubation was for 3 hr. at 30° C. in the presence and absence of 1 μg. estradiol-17β (E_2). α-Ketoglutarate (KG) was determined on deproteinized incubation mixtures. N is the number of duplicates; P, the probability (from t test) that the observed estradiol effect is due to chance. $P > 0.05$ is recorded as not significant (N.S.).

TABLE 3

ESTRADIOL-SENSITIVE ISOCITRIC DEHYDROGENASE IN HUMAN BREAST CANCER

Method	Total specimens	Number estradiol-sensitive	Per cent
Spectrophotometric	22	9	40.9
α-Ketoglutarate	14	5	35.7
Both methods	36	14	38.9

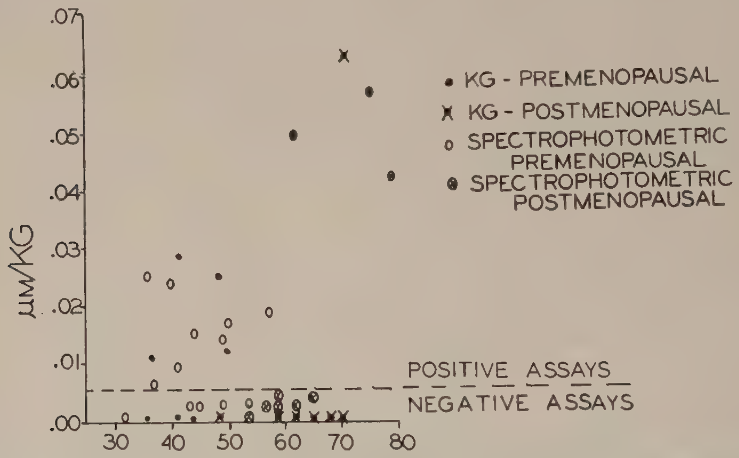

FIGURE 2. Age distribution of estradiol sensitivity in breast cancer. The incubation conditions were those given in the legend to TABLE 2. The ordinate represents the additional ketoglutarate due to the presence of estradiol.

that 38.9 per cent of the mammary cancers examined are sensitive *in vitro* to estradiol-17β, while Pearson *et al.*[14] report that 44 per cent of 75 premenopausal women who were oophorectomized achieved benefit from this procedure, presumably as a result of *in vivo* estrogen sensitivity.

FIGURE 2 shows the distribution by age of the enzymatic activity. There is a tendency for older women, if the mammary tissue is sensitive at all, to have a high degree of sensitivity. However, there is no striking effect of age or menstrual status on the occurrence of *in vitro* sensitivity. There was no correlation between enzymatic activity and the microscopic appearance of the tumor tissue except for a tendency to have higher estradiol sensitivities in the more cellular tumors, provided the tumor was sensitive at all. However, the degree of cellularity was not related to the qualitative presence of hormonal sensitivity.

Estradiol-Sensitive Enzyme in Mammary Tissue

When estradiol sensitivity was found in many mammary cancers, a systematic study of nonneoplastic human mammary tissue was carried out. Although mammary tissue from healthy individuals was not available, histologically

TABLE 4

ESTRADIOL-SENSITIVE ISOCITRIC DEHYDROGENASE IN HUMAN MAMMARY TISSUE

Method	Total specimens	Number estradiol-sensitive	Per cent
Spectrophotometric	25	12	48.0
α-Ketoglutarate	7	3	42.9
Both methods	32	15	46.9

normal tissue from mastectomy specimens was used. The breast tissue had been removed because of the presence of benign or malignant tumors.

TABLE 4 summarizes these studies to date. The fact that only 46.9 per cent of the specimens examined showed *in vitro* sensitivity was unexpected. Unless some other limiting factor is operating, all normal breast tissue should be sensitive to estradiol. The sensitivity of the mammary tissues studied was not related to age or menstrual status, and no relationship to cellularity or other microscopic appearance was noted. Enzymatic assay of tissue and an associated carcinoma was possible in 22 patients. In 13, both tissues failed to show sensitivity. In 7, both tissues showed *in vitro* hormonal sensitivity. In 2 patients no detectable sensitivity was observed in the tumor, although the associated mammary tissue was sensitive. To date, an enzymatically sensitive tumor has not been found in mammary tissue that does not exhibit sensitivity. No rank correlation was observed between enzyme levels in the breast and tumor.

Significance of the Absence of In Vitro Sensitivity

The separation of breast cancers into those types that are sensitive *in vitro* and those that are not is a qualitative separation based on the validity of the absence of the estradiol-sensitive system. The presence of an inhibitor in homogenates, an excess of endogenous estrogen, or a missing cofactor would result in a misleading negative assay.

FIGURE 3 shows an experiment done to test these three possibilities. An inactive breast homogenate is incubated in an appropriate optical system, and no estradiol effect on the rate of production of DPNH is noted. After a time, dialyzed hormonally sensitive placental homogenate is added to the same incubating systems. Prompt stimulation is apparent in the estradiol-containing cuvette, demonstrating that no inhibitor was present in the system and that no dialyzable cofactor limits the system. If excess endogenous estrogen were present in the breast homogenate, it should have been manifested by a higher initial rate of DPNH formation in both cuvettes, and no difference should have been observed in the presence or absence of added estradiol after addition of placental extract. Of course, the experiment is subject to the criticism that the mammary and placental systems may be different and have different requirements.

It might be postulated that the estrogen-sensitive enzyme system is found in mammary cancer because of the presence of normal mammary tissue contained

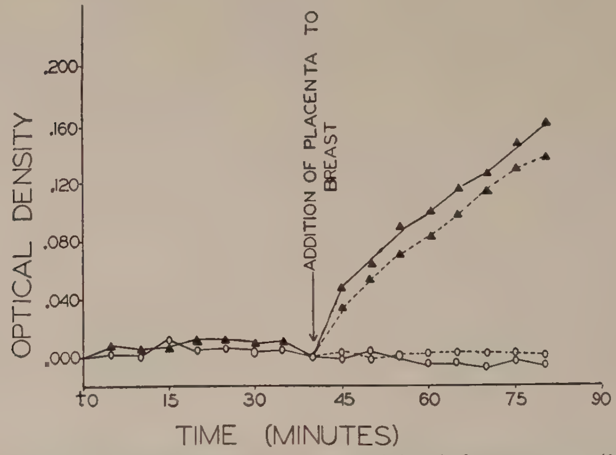

FIGURE 3. Test for the possible presence of (1) an inhibitor in homogenates, (2) an excess of endogenous estrogen, or (3) a missing cofactor. The incubation system consisted of 0.30 ml. mammary supernatant known not to be hormonally sensitive, 0.006 M phosphate buffer *p*H 6.8, 0.006 M $MgCl_2$, 0.001 M Versene, 0.002 M isocitrate, and 0.00035 M DPN in a final volume of 1.0 ml. Two cuvettes contained this system, and 1 μg. estradiol was added to the second. The ordinate is the optical density at 340 μM. At the point indicated by the arrow, 0.30 ml. placental supernatant fraction was added. The rate is compared with that from placental supernatant in the absence of mammary supernatant in separate cuvettes. Symbols: -▲- = breast homogenate with estradiol; -○- = breast homogenate without estradiol; --▲-- = placental homogenate alone with estradiol; and --○-- = placental homogenate alone without estradiol.

in the tumor. This suggestion is consistent with the observation that, to date, no *in vitro* sensitive mammary cancer has been observed associated with insensitive mammary tissue. However, this possibility may be eliminated since *in vitro* sensitivity has been found in three metastatic samples of breast cancer.

The presence of detectable levels of estradiol-sensitive enzyme in homogenates of mammary tissue and associated cancers seems to depend on some unknown systemic factor. A tumor may or may not exhibit sensitivity if this factor permits the associated breast to exhibit sensitivity. The effects of prolactin, growth hormone, and gonadotropin on this system are under study.

Clinical Correlation

The presence in estrogen-stimulated end organs of an enzyme system that is stimulated *in vitro* by low concentrations of estradiol may well have physiological significance.

The stimulation of citrate utilization engendered by estradiol would yield extra energy to make hormone-induced growth possible. If this enzymatic system were the locus of estrogen action at cellular level, it might be expected that tumors sensitive *in vivo* to estrogen might show *in vitro* stimulation by estradiol, while tumors not estrogen-sensitive *in vivo* would not show *in vitro* sensitivity.

To date, it has been possible to carry out enzymatic assays and oophorectomy in only four patients:

M. B. H.,[10] a 39-year-old female, had a radical mastectomy for infiltrating duct carcinoma in March 1955. In 1956 recurrent skin nodules appeared, and no detectable *in vitro* effect of estradiol was noted in biopsied tumor. Oophorectomy was done 1 month later, followed by steady objective progression of disease.

M. D.,[10] a 45-year-old female, had a radical mastectomy for infiltrating duct carcinoma in 1953. Local recurrence in 1955 was treated by X ray. In November 1955, bilateral oophorectomy was done. Immediately thereafter new lesions appeared and there was no regression in any lesion. Enzyme assay of biopsied tumor in July 1956 showed a barely detectable estradiol effect of doubtful significance.

A. M. D., a 38-year-old female, had a radical mastectomy in 1953 for scirrhous carcinoma. Local recurrence in 1956 gradually progressed until May 1957, at which time multiple skin nodules and a large malignant ulcer were present on the chest wall. Bilateral oophorectomy at this time was followed by subjective improvement and marked objective regression of the ulcer and disappearance of skin nodules. This striking remission lasted 7 months, when a supraclavicular node appeared, and the patient began to complain of such severe backache that she was confined to bed. X-ray examination of the previously negative spine demonstrated osteolytic metastases. Enzymatic assay of the metastatic node demonstrated (TABLE 2) the unequivocal presence of *in vitro* estradiol sensitivity. Bilateral adrenalectomy in January 1958 was followed by prompt disappearance of bone pain and by ambulation, but sufficient time has not elapsed to permit an objective evaluation of the results of this therapy. However, there is no question that the tumor had shown regression following the removal of ovarian estrogen in 1957.

E. M. J., a 48-year-old female, had an inoperable breast carcinoma with lenticular metastases. Biopsy in January 1958 revealed a diagnosis of infiltrating duct carcinoma, and the tumor (TABLE 2) showed definite *in vitro* sensitivity to estradiol. Bilateral oophorectomy in January 1958 was followed within a month by definite objective regression of the tumor, as well as striking subjective improvement.

The agreement between *in vivo* and *in vitro* behavior observed in these four cases may be coincidental. Obviously many more cases must be studied to establish a correlation of *in vitro* and *in vivo* estrogen sensitivity.

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Discussion of the Paper

QUESTION: Have you had a chance to study the human breast cancers in their metastatic form to see whether the same differences exist there as you saw in the primary site?

HOLLANDER: We have had the opportunity to study only about six examples of metastatic tumor. In three of these we found the enzymatic system we described, and this finding now eliminates one of the possibilities of explaining this curious relationship between the cancer and the breast. Our activity is not due to intramixed normal tissue in the cancer. It is present in metastatic tissue.

HORMONAL PATTERNS AS INDICES OF TUMOR ACTIVITY

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Under certain conditions steroid hormones may inhibit the growth of three types of human tumors. Breast cancer may respond to androgens, to estrogens and, to a limited extent, to corticoids; prostatic cancer may respond to estrogens; and leukemia may respond to corticoids. In general, steroid treatment is not effective in all cases and, even when a patient responds favorably, the duration of effectiveness is relatively short. Further difficulties involve undesirable side effects, which may be so severe as to restrict or prevent continued treatment. For these reasons it is desirable to find new steroidal agents that control tumor growth in a higher percentage of patients, that have a more sustained action, and that have minimum or no undesirable side effects.

In advancing this investigation, we are faced with at least two important basic questions. Do superior therapeutic steroidal agents exist and, if they do, how do we go about finding them? The answer to the first question is achieved with a blend of optimism and argument by analogy. To bring our arguments about steroids into sharper focus, we may examine some generalities concerning their biological action. Steroids, like many pharmacological agents, frequently possess not one but multiple actions. Thus, androgens influence sex-specific tissue (such as prostate, seminal vesicles, and penis), protein metabolism, electrolyte balance, and even some parts of the nervous system. Corticoids, as another example of different actions residing in the same molecule, influence carbohydrate and protein metabolism, and they control, among other actions, water and electrolyte balance. Of particular importance is the fact that, in both hormone series, chemical manipulations of the molecule have not only lead to major quantitative changes in activity, but one type of activity has been significantly and dramatically modified in comparison with another. Introduction of a 9α-fluoro substitution in cortisol increases the metabolic activity somewhat, but enormously increases the ability to retain sodium and excrete potassium. Introduction of a 16α-hydroxyl group into 9α-fluoroprednisolone changes the steroid from an intense sodium retainer to one that actually causes sodium excretion, while the metabolic activity of the compound is effected only minimally.

Separation of activities in the steroid series is not limited to the corticoids, but also is found in modified androgens. In this latter series, compounds have been synthesized that exhibit different relative potencies on the seminal vesicles and prostate on the one hand and the levator ani on the other. Removal of the C-19 carbon from testosterone, forming 19-nortestosterone, results in a steroid with an exaggerated response on the levator ani, as compared to its effectiveness of the seminal vesicle and prostate. Even more striking is the 17α-ethyl derivative of 19-nortestosterone, which has an even more intense levator ani activity, together with a reduced action on the seminal vesicle and prostate gland.

We now return to our first question and our argument by analogy. Simply stated, the proposition says that, since steroids, possessing multiple biological activities, can be so modified that a given activity may be increased or decreased relative to a second biological property, it follows that the possibility exists that antitumor activity of a steroid could be increased significantly in relation to other biological properties.

Our second question assumes that steroids may be so modified as to be more effective tumor agents than those now available in relation to the undesirable properties, and asks how one recognizes such an agent. One procedure might involve toxicological studies followed by clinical trial. This method may involve tedious trials and, unless large series of patients are employed on each compound, only dramatically effective agents can be detected. Those agents showing minimum effects would most often be missed and many clues lost. Another method for pursuing this problem could be the introduction of animal tests aimed at correlating activities at this level with clinical effectiveness so that, after a reasonable experience had been accumulated, intensive animal screening could in fact predict effectiveness in the patient. This methodology is the basis of our present program. It may be added at this point that this procedure is in wide use in the screening of steroids for their effectiveness in collagen diseases. Effective correlation between thymolytic and anti-inflammatory activity in rats and clinical effectiveness in humans has been established with reasonable certainty.

A third important question must be asked. What type of steroids do we expect will be effective against the various tumors? Certain facts and hypotheses can guide us in this respect, but it must be realized that these are valuable only for the purpose of further experimentation and should not be viewed as a rigid set of principles.

Breast cancer in premenopausal patients may be stimulated by estrogens and by growth hormone. Androgens and, to some extent, corticoids have antitumor effects. On this basis we can suggest the type of steroids to be used experimentally in this state. Potent antiestrogenic steroids (those interfering with the peripheral action of estrogens) may be of real value. Androgens are effective antibreast cancer agents, but the masculinization obviously is undesirable. Since androgens in general have an "anabolic action" as well, is it possible that the antitumor effect is really correlated with this latter property? To test this possibility, clinical trials should be done with those agents having high anabolic activity and relatively low androgenic activity. There is a further possibility: that the antitumor activity is a property independent of both the androgenic and anabolic properties of the molecule. The fact that corticoids do cause some favorable objective effects in breast cancer indicates the importance of studies oriented toward the possibility that there exists an antitumor factor quite aside from other activities of these compounds.

If growth hormone plays a true stimulating role in breast cancer, as preliminary experiments of some investigators have indicated, then it follows that agents that inhibit the peripheral action of this pituitary hormone could be of therapeutic importance. By the same token, agents having a selective and effective inhibiting action on the pituitary gland with respect to gonadotrophic hormone and ACTH production may be important.

In postmenopausal breast cancer, both estrogens and androgens as well as corticoids have been employed. The arguments in the androgen and corticoid series are those already advanced. With respect to the estrogen series, it remains to be established whether the antitumor activity can in fact be separated from the estrogenic activity of a steroid.

The problem of new steroids for improved therapy in prostatic cancer involves the estrogens and antipituitary materials, as already discussed, and a further type of agent, nonestrogenic antiandrogens. Since in certain phases of this disease androgens stimulate the growth of these tumors, intense antiandrogens would be expected to be valuable agents. Actually the estrogens have additional undesirable properties. Thus, the search is for effective antiandrogens that have no (or at least very minimal) complicating activities.

Therapy of leukemia by corticoids is well established; here again the problem of separation of activities confronts us. Is it possible that corticoid-type steroids have an antileukemic activity distinct from the established metabolic and electrolyte activities? The answer can come only from further investigations.

The specific program has three dimensions: the general biological profile of steroids, steroids as antitumor agents in experimental animals and, finally, clinical studies in humans. At the present time isolated facts are available from all three types of steroid activity evaluation, but intercorrelations are sparse. Other contributors to this monograph deal with tumor screening and clinical trials. The concern of our group* is the biological evaluation of the steroids. To this end we are concerned in the broadest sense with any and all dimensions of steroid activity because, at this stage of development, we cannot judge which biological index will correlate with antitumor effectiveness in humans.

The biological testing program has been set up with certain philosophical limits. Tests are classified as preliminary and quantitative. Preliminary assays are performed with two objectives. First, the compounds are separated into active and inactive groups, the dividing line being 5 per cent activity of an arbitrary standard. Indeed, if a steroid is judged to be active, a preliminary estimate of relative potency compared to the standard is made on the basis of this limited assay. On the basis of this preliminary estimate a quantitative assay is performed, which gives a measure of activity with an error of ±25 per cent at $P = 0.05$. Unfortunately, not all types of steroid activity can be measured with this accuracy by existing methods.

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* This group is the Subcommittee on Biological Activities of the Endocrine Panel of the Cancer Chemotherapy National Service Center, Bethesda, Md., and consists of Ralph I. Dorfman, Chairman; James H. Leathem, Roland K. Meyer, Preston L. Perlman, Robert O. Stafford, and Max X. Zarrow. This group operates as an advisory body to the Staff of the Cancer Chemotherapy National Service Center, which includes Joseph Leiter, Erwin P. Vollmer, Arthur G. Hilgar, Nathan Mantel, and Seymour Lieberman.

Bioassays are now being performed in a routine manner for following types of biological activity:

Bioassay	Animal	End point
Androgenic-myotrophic	Castrated male rat	Seminal vesicle Prostate Levator ani
Antiandrogenic and antimyotrophic	Castrated male rat	Seminal vesicle Prostate Levator ani
Uterotrophic	Immature female mouse	Uterus
Antiuterotrophic	Immature female mouse	Uterus
Thymolytic and anti-inflammatory	Adrenalectomized rat	Thymus granuloma
Antagonists to thymolytic and anti-inflammatory agents	Adrenalectomized rat	Thymus granuloma
Liver glycogen deposition	Adrenalectomized rat	Liver glycogen

The following additional bioassays are in the developmental stage:

Bioassay	Animal	End point
Progestational	Rabbit	Uterine histology and carbonic anhydrase content
Pituitary inhibition (ACTH)	Guinea pig	Cortisol in urine
Eosinophil	Adrenalectomized mouse	Blood eosinophil count
Electrolyte	Adrenalectomized rat	Urinary sodium and potassium

A final group of tests soon to be developed for the program includes the following:

Bioassay	Animal	End point
Pituitary inhibition (gonadotropins)	Parabiotic rats	Gonad weights
Antiprogestational activity	Rabbit	Uterine histology and carbonic anhydrase content
Pituitary inhibition (ACTH)	Rat	Corticosterone content of adrenals
Antigrowth hormone	Rat (plateaued female or hypophysectomized)	Body growth

When all these tests are effectively in operation and data are obtained on a variety of compounds, plus adequate clinical trials on a reasonable number of active compounds, it is not unlikely that an effective animal screen could be evolved for finding compounds having more desirable properties for human tumors than are available at the present time.

Summary

Patients with breast and prostatic tumors and leukemia may under certain conditions be treated effectively with steroid hormones and related compounds. This therapy has limited value because of the lack of effectiveness in many patients, the short duration of therapeutic effect, and serious undesirable side effects. A program has been instituted to find more effective steroids by extensive bioassays of compounds in experimental animals, in tumor-bearing animals, and clinical trials. By correlating the effects of the compounds in experimental animals and in cancer patients it is hoped that an effective animal screen can be developed that will predict the effectiveness of these agents in human disease.

SCREENING HORMONAL AGENTS FOR ANTICANCER ACTIVITY IN MAN

By Albert Segaloff

The Endocrine Research Division of The Alton Ochsner Medical Foundation and The Department of Medicine of Tulane University School of Medicine, New Orleans, La.

There are, of course, many human tumors not yet adequately tested for their responsiveness to hormonal agents. The screening process in man therefore must be adapted to yield information as to whether a tumor is truly responsive. It is useless simply to treat patients in an unorganized fashion and hope for results so dramatic that they cannot fail to be recognized.

With the untested type of tumor, this stage of screening both tumors and hormonal agents becomes one of pioneering exploration. It is necessary to try a series of tumors of the type in question to see if they will respond favorably, usually selecting the best known and most potent available hormonal agent, or ablative procedures designed to produce profound changes in the hormonal internal milieu. This type of exploration is limited only by the scope of the investigator's imagination and the availability of patients with specific tumors and hormonal materials, although end points of activity sufficiently sensitive to show favorable effects, should they occur, must be determined.

However, once one is able to ascertain that a given tumor is responsive to changes in its hormonal environment, it is possible to set up an adequate screening method in man in the search for the most effective agents against the specific tumor. Such is the case for advanced breast cancer.

It may seem unnecessary to some but, since we are discussing screening methods, it is imperative to realize that, for maximum benefit, patients must be assigned to therapy groups by some method that cannot be manipulated, such as the drawing of consecutively numbered envelopes containing the patient assignments, based on an adequate random method. Wherever possible, the therapy should be dispensed by number, in form physically the same as the reference standard, that is, double blind. However, in the interest of good patient care, a means of breaking the code for a given patient must be at hand in case the patient's medical status should require it.

It is my own considered opinion that, when there is a known effective agent for treating a given type of malignancy, it does not add to the screening efficiency to include a control group chosen on a random basis. In addition to offering no statistical or actual advantage, this constitutes a real and unnecessary cruelty to the patient, who might well have responded favorably to the known agent. It is also unnecessarily cruel (and probably impossible) to prevent patients from receiving subsequent therapy of possible benefit in order to obtain longevity figures for a given therapeutic modality.

In devising an adequate screening system and applying it so that the greatest knowledge can be gained from treating the smallest number of patients, it is of utmost importance that the investigator be thoroughly versed in the natural history of the tumor in question. If not, he will only add to the mass of medical literature on dramatic response to therapy which is, in reality, only a measure of the usual course of the disease. Despite the general impression to the contrary, breast cancer, once started, does not proceed with measured cadence until a vital center is destroyed and the patient dies. These tumors have greatly varied rates of growth in different hosts and tend to proceed irregularly in their respective growth rates. Stated differently, breast cancer emulates the bear and the groundhog, going through periods of hibernation during which life and viability are maintained while growth and active function are not. The outstanding example of this is the patient who, after radical mastectomy and absence of signs, symptoms, and demonstrable evidence of cancer for as long as 20 or 30 years, suffers a pathological fracture of the femur which, at biopsy at the open reduction, shows tumor microscopically identical with that of the primary cancer. In such cases the tumor usually grows rapidly and, unless the patient responds well to other measures, further duration of life is very short. Here, then, is a tumor that lay dormant for years and then awoke abruptly and actively. While knowledge of a method for inducing such periods of quiescence would be of great help in the therapy of breast cancer, one must be wary of the trap of interpreting a spontaneous quiescent period as a response to therapy, or of initiating courses of therapy during periods of quiescence and interpreting the resulting stationary period as having been induced by the therapy. Therefore, with this disease it is imperative that we base our decisions concerning response to therapy on the objective criteria of induction of regression in other lesions previously shown to be advancing in the same manner.

It has been known for some time that castration of premenopausal women produces, in a substantial percentage of cases, regression of advancing breast cancer. Accordingly, patients who are either menstruating or not more than one year postmenopausal are castrated. When their good responses have run their course and the disease is again progressing, or when it can be shown that the disease has not been affected by the castration, these patients make very favorable subjects for the testing of new administrative hormonal agents. There is a reference standard compound, testosterone propionate which, our authorities agree, produces objective remission of the disease in about one quarter of the patients treated; it is thus possible to compare the effectiveness of our new therapeutic agents to that of this reference standard.

Accordingly, a procedure was devised whereby the patients are randomized onto the experimental therapy and the reference standard. Whenever possible, the new agent and the reference standard are studied in a double-blind fashion. Since there is a substantial body of evidence that the menopausal age of the patients has an effect, the patients are randomized into four groups depending upon their menopausal age. It has been said that when certain sites of metastases predominate they carry with them a poorer or better prognosis; patients were therefore randomized into three groups according to the predominating site of the metastases. Thus there were twelve groups set up according to menopausal age and predominant type of lesion before individuals were randomized between the reference standard and the experimental compound. The number of patients was so set that the compounds adjudged to be significantly less effective than the reference standard could be rejected forthwith, and the most promising agents, therefore, could be studied further.

Naturally, it is our hope that some highly effective agent will be found, so that it will not be necessary to carry out extensive studies to delineate small improvements. However, if agents very close in activity to the currently available agents are the best ones thus far found, they will be studied further so that the best can be ascertained. As results improve, such new and more effective agents will be substituted for the present reference standard, so that rejection of compounds as less effective will be possible with the use of less material to treat fewer patients. However, we shall then be looking for compounds of a higher order of activity to compete with this new, more effective standard that we have not yet attained.

In a screening procedure such as this, dosage of medication is an almost insurmountable problem of logistics which, in the last analysis, must be solved empirically. Where there is some known biological activity, such as androgenicity, dosage is equated as an approximate biological equivalent to the standard. The initial dosage for the other agents is, of necessity, set arbitrarily. It is important to point out that in all honesty, virilization from an androgen cannot be referred to as a side reaction. This is actually a reflection of the prime biological activity of androgens.

The selection of hormonal agents for screening against responsive human cancers presents several difficult problems. In our best known hormonally responsive tumor, advanced breast cancer, we have no known reliable guide in any of our animal or laboratory work that can be used unequivocally to select effective hormonal agents in advance. The few leads we possess have been pursued—namely, that potent estrogens and androgens are effective against advanced breast cancer. Heretofore, all of the effective agents have had a substantial lowering effect on the excretion of gonad-stimulating hormone in the urine, while the ineffective agents have failed to do so. This fact has been employed as a guide. However, since there is no animal tumor system of a high order of adequacy in predicting response, and since the very limited number of trials of varying agents carried out prevents certainty, it has been necessary to set up a screening program in man that would take advantage of any lead that appeared worth while. Accordingly, steroids are being screened against advanced breast cancer either because of their close chemical relationship to known effective androgens, their high order of effectiveness as androgens, their ability to inhibit gonad-stimulating hormone excretion, their possession of a unique and as yet untried chemical structure, their display of a unique pattern of biological activity in animals, or because they have been shown to be highly effective in any one of several animal tumor systems.

Acknowledgments

The protocol for screening hormonal therapy against advancing breast cancer now being used by the cooperative groups under the Cancer Chemotherapy National Service Center, upon which this discussion was based, was written by a committee that included myself as chairman, Delbert Bergenstal, George C. Escher, and Robert A. Huseby. We are grateful for statistical advice and help from Marvin Schneiderman and Nancy Brombacher of the staff of the Center.

SCREENING STEROIDS AGAINST A SPECTRUM OF TUMORS*

By C. Chester Stock and Kanematsu Sugiura

Sloan-Kettering Institute for Cancer Research and Sloan-Kettering Division, Cornell University Medical College, New York, N. Y.

The title of this report might better have been stated as the screening of a spectrum of tumors against steroids, for, in effect, that is what we have done. In answer to Albert Segaloff's inquiring statement in the preceding paper, we do not have a spectrum of tumors containing some responsive to the steroids that would affect breast cancer. In all probability we must look for interesting tumors of that type among those that J. Furth has been making available (see the first paper in Part V). The tumors that are truly hormone dependent are of greatest pertinence in steroid screening.

Several years ago, when cortisone became available, Sugiura began to test steroids against a number of tumors. Even with our limited personal knowledge of endocrinology, we had no great expectation that the transplantable tumors in Sugiura's spectrum would be particularly susceptible to the steroids, regardless of the category, whether androgenic, estrogenic, progestational, or corticoid. However, as some of the contributors have indicated, the tumors that do seem to be hormone responsive possess characteristics that are not the most convenient for screening purposes. Therefore it would be a shame to neglect readily available tumors that could be relatively easily studied if by chance any of them showed a response that could be useful in selecting steroids or other hormones for testing in man.

Since most of the pertinent data already have been reported,[1-5] the earlier steroid-spectrum studies will be reviewed but briefly. The four steps involved in the testing procedure are as follows: (1) weigh individual mice (5/compound), (2) implant tumor pieces subcutaneously, (3) start subcutaneous injections of steroid 24 hours later and continue once daily for 7 days, (4) weigh mice and measure tumors in 2 dimensions with calipers at weekly intervals.

The degree of inhibition of the growth of the solid tumors was graded according to the following scheme: +++, complete destruction of tumors (complete regression of more than 50 per cent of tumors in the treated animals); ++, marked inhibition (failure to grow, or growth to approximately one fourth of the average diameter of the controls); +, moderate inhibition (tumor growth from one fourth to one half of the diameter of the controls); ±, slight inhibition (tumor growth from one half to three quarters of the diameter of the controls); −, no effect (tumor growth to three quarters or more of the diameter of the controls).

The degree of inhibition of the growth of the ascites tumors was graded according to the following scheme: +++, complete inhibition or destruction of ascites; ++, marked inhibition, indicating no abdominal distention (no gross ascites); +, moderate inhibition, indicating slight abdominal distention (the fluid volume increases to about one fourth the volume of the controls, 1 to 5 cc.); ±, slight inhibition, indicating moderate abdominal distention (the fluid volume increases to about one half the volume of the controls, 6 to 15 cc.); −, no effect, indicating marked abdominal distention (the fluid volume increases to three fourths or more of the controls, 16 to 30 cc.).

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* The work reported in this paper was aided by a grant from the American Cancer Society, Inc., New York, N. Y.

In some animals with ascites the daily weight increase may be much less, owing to the toxic actions of treatment on the host rather than against the diminished ascites tumor. Survival of more than 50 per cent of the treated animals beyond 3 weeks was considered a marked effect. Under our experimental conditions the untreated control animals die in 10 to 14 days. This technique differs from that routinely used for other compounds in that subcutaneous rather than intraperitoneal injections were used.

TABLE 1 shows the influence of the route of injection on the inhibitory effects

TABLE 1
INHIBITION OF MOUSE LYMPHOSARCOMA: CORTISONE ACETATE INJECTED ONCE DAILY FOR SEVEN DAYS

Route	Dose (mg./kg.)	Effect
S.C.	12.5	+
S.C.	25	++
I.P.	25	−
I.P.	100	±
Oral	250	±

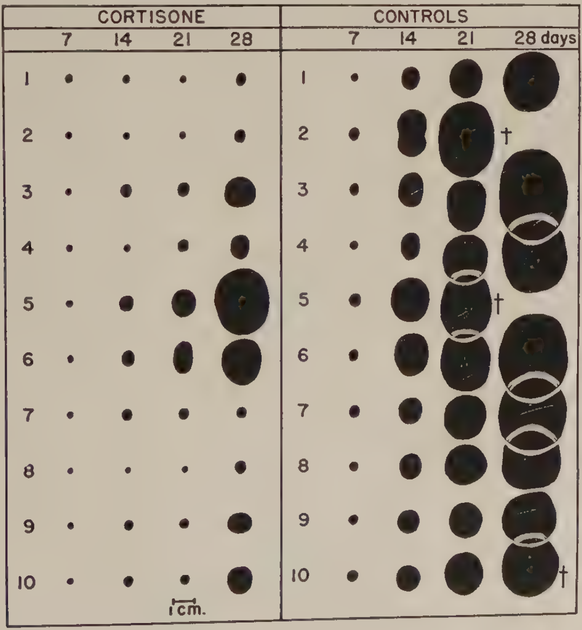


FIGURE 1. Effect of cortisone on osteogenic sarcoma in mice.

Table 2

Miscellaneous Steroids Tested Against Various Tumors*

Compound	Dose (mg./kg./day)	Tumor†															
		S 180	T 241	Ma 387	E 0771	GLS	MLS	PLS	ROS	WOS	Ca 63	Ca 1025	EC	EC (A)	LC	MM	W 256
3β-Hydroxy-Δ^{5}-pregnen-20-one	300	−	−		−		−		−		−						
	100		−		−		−			+		−	−				
21-Acetoxy-3β-hydroxy-Δ^{5}-pregnen-20-one	200–375				−		−	−	−	−		−				−	
	150	−	−		−												
Δ^{5}-Pregnene-3β, 17α, 20α (and β)-triol	500	−	−	−	T		−	±	−	−	−					−	
Δ$^{5,17(20)}$-Pregnadien-3β-ol	500	−	−				−	−	−	−		−					
3β-Hydroxy-16β-methyl-Δ^{5}-pregnen-20-one	500	−	−				−	±	− to +								
	375						−	−		−		±	−				
3β-Acetoxy-17α-hydroxyallopregnan-20-one	500				−			−	±	−			−			−	
	375		−		−			−		±	−						
3β-Acetoxy-Δ^{5}-norcholesten-24-one	500	−	−	−	−		−		±	−	−					−	
Δ^{5}-Cholenic acid-3β, 20-diol-20-lactone	500	−	−	−	−	−	−	±	−	−	−	±	−			−	
3β-Acetoxy-Δ^{5}-norcholesten-25-one	500	−	−	−	−		−		±	−	−					−	
3β-Acetoxy-16-methyl-Δ^{5,16}-pregnadien-20-one	500	−	−		−		−	−		−	−						
3β, 21-Diacetoxyallopregnan-20-one	375	−	−		−		−	−	−	−	±	−	−				
Δ^{1}-Androstene-3, 17-dione	375						−	−	−	±		−					
17β-Hydroxy-Δ^{4}-androsten-3-one	500	−	−		±		−	−		−	−	−	−				
Δ^{4}-Androstene-3, 17-dione	375	−			−		−	−	−	−	−	−		+		−	
Δ^{4}-Pregnene-3, 20-dione	500		−											−		−	
	375			−					−		−	−		−			
	250				−		−	−		−							
21-Acetoxyallopregnane-3, 20-dione	250	−	−		−		−	−	−	−	−	−	−				
21-Acetoxy-17α-hydroxy-Δ^{4}-pregnene-3, 20-dione	450		−				−	−		−							−
	250				−		−	−	−	−							
21-Acetoxy-Δ^{4}-pregnene-3, 20-dione	500	−	±	−	−		−		−	−	−	−	−	−			
17α, 20β-Dihydroxy-Δ^{4}-pregnen-3-one	375						−	−	−								
	250	−		−	−					−					−		

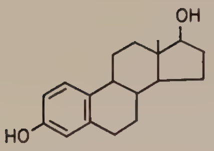
21-Acetoxy-Δ^4-pregnene-3,11-20-trione	250				++‡			T	+ to ++								
	125			−				++	++							−	
	75						−	+ to ++	++								
	50							± to ++									
21-Acetoxy-17α-hydroxypregnane-3,11,20-trione	375		−	−				−		−						−	
	200	−			−		−	−									
11β,17α,21-Trihydroxy-Δ^4-pregnene-3,20-dione	37.5	T	±		+ to ++		− to +	++	++	+ to ++			T	T			
	25		±		+		±	++	+			+		T	±		+
	12.5							− to +									

---

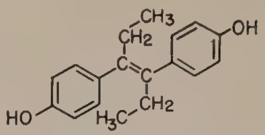
* Tumor growth retardation has been graded as follows: T = toxic; − = growth more than three-fourths the average diameter of the controls; ± = growth from one-half to three-fourths the average diameter of the controls; + = growth from one-fourth to one-half the average diameter of the controls; ++ = no growth, or growth to one-fourth the average diameter of the controls; and +++ = destruction of tumor.

† Explanation of tumor abbreviations used in TABLES 2, 3, and 4: EC (A) and S 180 (A) = the ascitic forms of the Ehrlich carcinoma and the Crocker sarcoma 180; T 241 = sarcoma T 241; Ma 387 = sarcoma Ma 387; E 0771 = mammary adenocarcinoma E 0771; GLS, MLS, and PLS = the Gardner, Mecca, and Patterson lymphosarcomas, respectively; ROS and WOS = Ridgway and Wagner osteogenic sarcomas, respectively; Ca 63 = carcinoma 63; Ca 1025 = carcinoma 1025; EC = Ehrlich carcinoma; LC and BC = Lewis lung and bladder carcinomas, respectively; MM = Harding-Passey melanoma; W 256 = Walker 256, Walker carcinoma 256, and Walker carcinosarcoma 256; MC = Miyono adenocarcinoma; G 26 = glioma 26; FVT = Friend virus tumor (leukemia); JRS = Jensen rat sarcoma; MRLS = Murphy rat lymphosarcoma; FRC = Flexner-Jobling rat carcinoma.

‡ One test toxic.

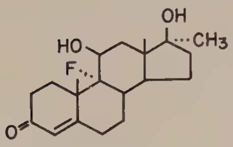


$\Delta^{1,3,5(10)}$-ESTRATRIENE-3,17β-DIOL

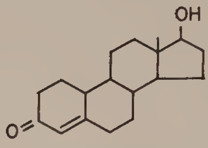


DIETHYLSTILBESTROL

ESTROGENS

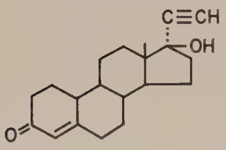


9α-FLUORO-11β, 17β-DIHYDROXY-17α-METHYL-Δ^4-ANDROSTEN-3-ONE

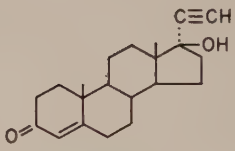


17β-HYDROXY-19-NOR-Δ^4-ANDROSTEN-3-ONE

ANDROGENS

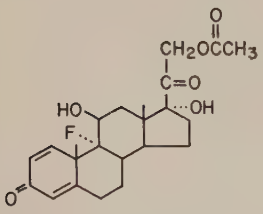


17β-HYDROXY-19-NOR-Δ^4-PREGNEN-20-YN-3-ONE

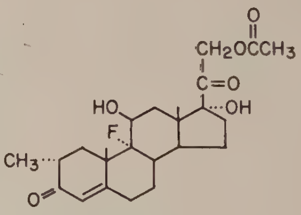


17β-HYDROXY-Δ^4-PREGNEN-20-YN-3-ONE

PROGESTOGENS



9α-FLUORO-21-ACETOXY-11β, 17α-DIHYDROXY-$\Delta^{1,4}$-PREGNADIENE-3,20-DIONE



9α-FLUORO-21-ACETOXY-11β, 17α-DIHYDROXY-2α-METHYL-Δ^4-PREGNENE-3,20-DIONE

CORTICOIDS

FIGURE 2

of cortisone on a mouse lymphosarcoma. In a test against the tumor spectrum, cortisone was found effective against several osteogenic sarcomas and lymphosarcomas in the mouse.[3, 4] FIGURE 1 illustrates the type of effect observed with cortisone on the Wagner osteogenic sarcoma. A check of the literature revealed a prior observation of the effectiveness of cortisone against a mouse lymphosarcoma.[6] An extension of our studies revealed that 3 steroids closely related to cortisone also were active, while many others were inactive

Selected Steroids Tested in a Tumor Spectrum*

Compound	Dose (mg./kg./day)	Tumor†																					
		S 180 (A)	S 180	T 241	Ma 387	E 0771	MC	MLS	ROS	WOS	Ca 63	Ca 1025	EC	EC (A)	BC	LC	MM	G 26	FVT	JRS	MRLS	W 256	FRC
Diethylstilbestrol	10	− to ++	−	±	−	−	±	T	− to +	++	−	−	−	−	±	±	+	−	+	−	T	−	−
$\Delta^{1,3,5(10)}$-Estratriene-3,17β-diol (17β-Estradiol)	40	−	−	−	± to +	−	−	±	±	T	−	−	−	−	±	−	−	+	++	−	−	±	−
17β-Hydroxy-19-nor-Δ^4-androsten-3-one (19-Nortestosterone)	20	−	−	−	−	−	−	−	−	−	−	−	±	±	−	−	−	−	++	−	−	±	−
	500	±	−	−	−	−	− to ±	−	±	−	−	++	− to ±	±	±	−	T	−	±	±	−	±	−
9α-Fluoro-11β,17β-dihydroxy-17α-methyl-Δ^4-androsten-3-one (Halotestin)	375	−	−	−	−	±	−	−	−	−	−	−	±	−	−	−	−	−		−	−	−	−
17β-Hydroxy-Δ^4-pregnen-20-yn-3-one (17α-Ethynyltestosterone)	375	±	−	−	−	−	−	−	−	−	−	−	−	−	±	−	−	−	±	−	−	± to ++	−
17β-Hydroxy-19-nor-Δ^4-pregnen-20-yn-3-one (17α-Ethynyl-19-nortestosterone)	500	−	−	−	−	−	±	−	−	−	±	−	±	−	±	−	−	−	± to +	−	−	−	−
9α-Fluoro-21-acetoxy-11β,17α-dihydroxy-$\Delta^{1,4}$-pregnadiene-3,20-dione (9α-Fluoro-Δ^1-dehydrohydrocortisone acetate)	12.5	+‡	−	T	±	++	++	−	++	++‡	++‡	+	−	T	±	−‡	T	+ to ++	±‡	T	T	T	T
	6.25			T		++		−	++		++	−		+++			±*			T	T	T	T
	3.0					+			++											−	− to ±	+ to ++	T
9α-Fluoro-21-acetoxy-11β,17α-dihydroxy-2α-methyl-Δ^4-pregnene-3,20-dione (9α-Fluoro-2α-methylhydrocortisone-21 acetate)	37.5					++			++		++								++				
	25.0	++	− to ±	− to ±	− to ±	+ to ++	± to +	−	++	++	++	− to +	+	± to +	− to ±	− to ±	±‡	− to +	± to ++	+‡	T	T	T
	10–12.5	−				±			++	T	++						++				++	T	T
	5.0																			−	± to ++	T	T
17α,21-Dihydroxy-Δ^4-pregnene-3,11,20-trione (Cortisone)	37.5	−	+	+	−	+	−	++	++	++	+	±	±	−	−	−	−	±	±	−	±	−	−
	25.0	−					±	− to ±	++	++	+	±											

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* Tumor growth retardation graded as in TABLE 2.
† Tumor abbreviations are the same as in TABLE 2.
‡ One test toxic.

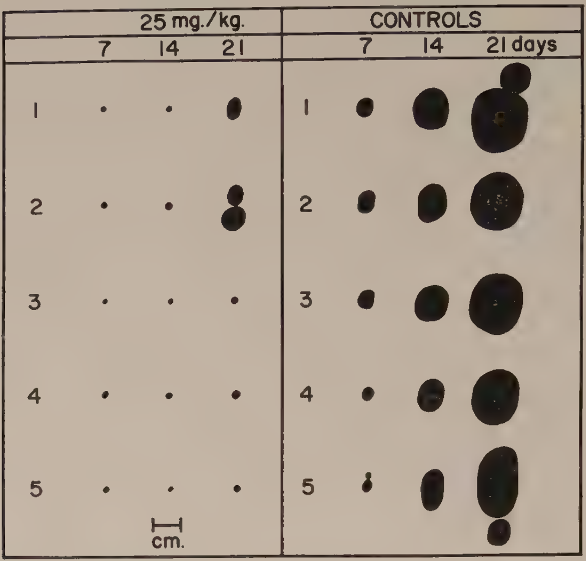

FIGURE 3. Effect of hydrocortisone, 9α-fluoro-2α-methyl-21 acetate on carcinoma 63 in mice.

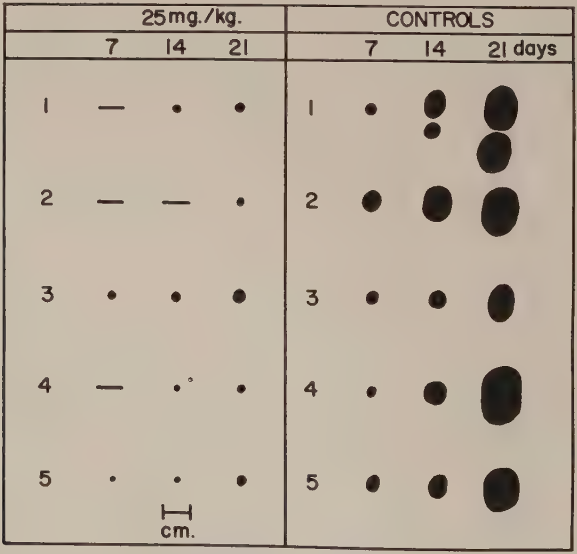

FIGURE 4. Effect of hydrocortisone, 9α-fluoro-2α-methyl-21 acetate on Wagner osteogenic sarcoma in mice.

at higher doses (TABLE 2) thus making apparent certain relationships of activity to structure.[5]

With this background we may consider the results obtained in challenging the spectrum of tumors with 8 selected steroids. These steroids, comprised of

TABLE 4

REPRESENTATIVE DETAILED DATA ON SEVERAL CORTICOIDS*

Compound	Dose mg./kg./day	Tumors† and results											
		E 0771			Ca 63			WOS			ROS		
		Wt. chg.	Deaths	Grading	Wt. chg.	Deaths	Grading	Wt. chg.	Deaths	Grading	Wt. chg.	Deaths	Grading
9α-Fluoro-21-acetoxy-11β,17α-dihydroxy-$\Delta^{1,4}$-pregnadiene-3,20-dione (9α-Fluoro-Δ^1-dehydrohydrocortisone acetate)	25									T	$\frac{-0.5}{+4.5}$	$\frac{2}{5}$	++
	12.5		$\frac{7}{10}$	T	$\frac{-3.5}{+7.0}$	$\frac{2}{5}$	++‡	$\frac{0.0}{+5.0}$	$\frac{1}{5}$	++‡	$\frac{-2.0}{+4.5}$	$\frac{2}{5}$	++
	6.25	$\frac{-1.5}{+2.5}$	$\frac{2}{10}$	++	$\frac{-1.5}{+2.5}$	$\frac{3}{10}$	+ to ++				$\frac{-1.0}{+4.5}$	$\frac{1}{10}$	++
	3.0	$\frac{-2.5}{+1.5}$	$\frac{0}{5}$	+							$\frac{+1.5}{+4.5}$	$\frac{1}{5}$	++
9α-Fluoro-21-acetoxy-11β,17α-dihydroxy-2α-methyl-Δ^4-pregnene-3,20-dione (9α-Fluoro-2α-methylhydrocortisone-21 acetate)	37.5	$\frac{-1.0}{+3.0}$	$\frac{2}{5}$	++	$\frac{-1.5}{+3.5}$	$\frac{0}{5}$	++				$\frac{-2.0}{+2.0}$	$\frac{1}{5}$	++
	25	$\frac{-1.5}{+3.0}$	$\frac{3}{10}$	+ to ++	$\frac{-1.0}{+3.5}$	$\frac{1}{15}$	++	$\frac{-1.5}{+3.0}$	$\frac{1}{10}$	++	$\frac{-1.5}{+3.0}$	$\frac{2}{10}$	++
	12.5	$\frac{-0.5}{+3.0}$	$\frac{0}{5}$	±	$\frac{+1.0}{+3.5}$	$\frac{1}{5}$	++		$\frac{4}{5}$	T	$\frac{+0.5}{+2.0}$	$\frac{0}{5}$	++

* Tumor growth retardation graded as in TABLE 2.

† Tumor abbreviations are the same as in TABLE 2.

‡ One test toxic.

2 androgens, 2 estrogens, 2 progestogens, and 2 corticoids (FIGURE 2), were studied at the request of the Endocrinology Panel of the Cancer Chemotherapy National Service Center, Bethesda, Md., to determine whether any of the tumors would respond. The data are summarized in TABLE 3. Cortisone has been included for comparative purposes. Although it has not been possible to test all steroids against all tumors in repeated tests, we believe that added testing would not change the results obtained thus far. Except in the case of the corticosteroids, these data indicate no significant reproducible activity from the selected steroids. It will be noted that the steroids have been tested at doses considerably larger than those normally regarded as physiological. In the case of the active substances and others tested in doses below 100 mg./kg./day, the top doses used were those at which toxicity was observed. In FIGURES 3 and 4 the degree of retardation of tumor growth shown by 9α-fluoro-21-acetoxy-11β,17α-dihydroxy-2α-methyl-Δ^4-pregnene-3,20-dione at 25 mg./kg./day and 9α-fluoro-21-acetoxy-11β,17α-dihydroxy-$\Delta^{1,4}$-pregnadiene-3,20-dione is illustrated for the former compound tested against the Wagner osteogenic sarcoma and Carcinoma 63.

Representative detailed test data are presented in TABLE 4 for the two corticosteroids, which appear somewhat superior to cortisone in these tests, but a more careful comparison will be required to define the relative merits of the compounds.

In summary, we are very much in agreement with Furth in his belief that we must look elsewhere for tumors that will show a response to the noncorticoid steroids. There are tumors in the spectrum tested that are responsive to the corticoids. The two fluorinated corticoids have markedly retarded the growth of the two "osteogenic" sarcomas, of Carcinoma 63, and of mammary adenocarcinoma E 0771 and Miyono adenocarcinoma, with lesser activities against other tumors.

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TESTING OF HORMONES AND RELATED SUBSTANCES WITH HUMAN TUMORS GROWING ON THE CHICK EMBRYO*

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Introduction

The use of the chorioallantoic membrane (CAM) of the chick embryo for heterologous tumor growth began with the classic studies of Murphy (1912). Later the chick embryo was used to grow tumors from a variety of animals, including man. Stevenson (1918) reported the successful growth of a variety of mouse tumors. The chick embryo was used by Schrek and Avery (1937) to grow rat and rabbit tumors, and the serial passage in the egg of established transplantable human tumors was reported in 1954 by Dagg *et al.*

Human tumors grown on the CAM have been shown to respond to nitrogen mustard (Dagg, *et al.*, 1954), triazene (Dagg *et al.*, 1955), triethylenemelamine (Harris, 1956), and a number of compounds of microbial origin (Harris, 1957, and in his article elsewhere in this monograph).

These studies are concerned primarily with experimental chemotherapy utilizing hormones and related substances as test agents against human tumors growing on the CAM of chick embryos. Preliminary chemotherapy studies using hormones and related substances as test agents have indicated that such tumors may be useful in the evaluation of potential antitumor agents in these categories. Some preliminary results of our tests are presented in this report.

Procedures

Each egg to be implanted receives a single tumor fragment at 9 days of incubation. Observation for tumor growth occurs 4 days after implantation, and at this time tumor-bearing eggs are selected for the tests and single-dose injections of the test agents are made into the yolk sac. Evaluations are made 6 days after the beginning of therapy. These procedures are outlined by J. J. Harris in greater detail elsewhere in this volume. Four human tumor types are used in these studies: Toolan's epidermoid carcinoma (HEp 3), sarcoma (HS 1), and adenocarcinoma (HAd 1), and Skiff's bronchiogenic carcinoma (A 42).

Experimental

The results presented here are preliminary in the sense that they represent single tests of the agents against the various tumor types. Repeated tests of these agents must be made against the same tumor type before definite conclusions can be formed. Such tests are currently in progress in our laboratory.

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TABLE 1

EFFECT OF PROGESTERONE ON HUMAN TUMORS IN EGGS*

Tumor	Mortality		Embryo wt.		Av. tumor wt. (mg.)		Results (% inhib.)	
	C	T	C	T	C	T	Embryo	Tumor
HS 1..........	1/15	2/12	21	12	377	101	43	73
HEp 3........	4/12	2/6	20	13	1077	255	35	76
HAd 1........	0/8	0/6	22	12	429	147	45	66

* Testing was done with a dosage of 8.0 mg./egg.

Comparisons among the tumor types of the single-test results seem to indicate what one may expect when testing hormones and related substances against human tumors in the egg.

Progesterone. This agent was tested at 8.0 mg./egg against HS 1, HEp 3, and HAd 1. The results of these tests are indicated in TABLE 1. It will be noted that the degrees of tumor inhibition are similar for the 3 tumor types. The inhibition of embryo growth is similar in the 3 cases. Although these are preliminary results, a certain degree of antitumor specificity appears to be indicated. Results from testing other types of agents have indicated a total lack of antitumor specificity in many instances; that is, the degrees of inhibition of embryo growth and of tumor growth were comparable. We are currently testing progesterone at lower dose levels to determine whether or not a fair degree of tumor inhibition can be retained without appreciable growth inhibition of the embryo.

Cortisone. Three of the human tumor types were used in the preliminary testing of cortisone (TABLE 2). The tests against HEp 3 and A 42 utilized 2.5 mg./egg of cortisone, and a dose level of 2.0 mg./egg was used against HAd 1. It will be noted that cortisone appears to affect HEp 3 slightly more than it affects the other tumor types. The embryos bearing HEp 3 tumors appeared to be somewhat more affected by cortisone than embryos bearing other tumor types. Reasons for this are not yet apparent; however, it has been our observation that effects of a given agent at the same dose levels on embryos bearing the same tumor type may sometimes fluctuate among the repeated tests. This type of response to drugs or agents is not peculiar to this type of host.

Methyltestosterone. The results of tests using methyltestosterone against

TABLE 2

EFFECT OF CORTISONE ON HUMAN TUMORS IN EGGS

Tumor	Dose (mg./egg)	Mortality		Av. embryo wt. (gm.)		Av. tumor wt. (mg.)		Results (% inhib.)	
		C	T	C	T	C	T	Embryo	Tumor
HEp 3...........	2.5	7/24	2/20	22	14	777	356	36	54
A 42.............	2.5	2/8	2/6	17	17	1187	759		36
HAd 1...........	2.0	0/8	0/6	22	19	429	301	14	30

TABLE 3

EFFECT OF METHYL TESTOSTERONE ON HUMAN TUMORS IN EGGS

Tumor	Dose (mg./egg)	Mortality		Av. embryo wt. (gm.)		Av. tumor wt. (mg.)		Results (% inhib.)	
		C	T	C	T	C	T	Embryo	Tumor
HS 1.	16	1/8	3/6	22	18	453	364	18	20
HEp 3	16	6/10	2/6	18	16	834	308	11	63
	2	1/7	3/7	18	20	405	776		
HAd 1.	8	0/8	1/6	22	20	429	245	9	43

3 human tumor types in eggs are indicated in TABLE 3. This agent was tested against HS 1 and HEp 3 at a dose level of 16.0 mg./egg. The failure of HS 1 to respond to this dose of methyltestosterone is contrasted with the positive response of HEp 3. This agent was also tested against HEp 3 at a considerably lower dose level, 2.0 mg., egg. When the results of testing the 2.0 mg. and the 16.0 mg. dose levels of methyltestosterone against HEp 3 are compared, it appears that the 2.0 mg. dose stimulates growth of the tumor, while the 16.0 mg. dose inhibits tumor growth. Similar observations have been made in cases not reported here. As a result of such observations, we have begun testing hormones and related substances at various dose levels. Although the results are not particularly impressive, HAd 1 gave a positive response to methyltestosterone at a dose level of 8.0 mg./egg.

Testosterone propionate. This agent was tested against 3 human tumor types at a dose level of 20.0 mg./egg. The results are indicated in TABLE 4. It can be seen that HS 1 was inhibited to a slightly greater extent than either A 42 or HAd 1. Tests are currently in progress to determine whether the most effective dose level of testosterone propionate has been reached.

When the responses of these human tumors to testosterone propionate are compared with their responses to methyltestosterone, the most striking response is that of HS 1 (TABLES 3 and 4). Negative results were obtained when methyl testosterone was tested against HS 1, while positive results were obtained in the test using testosterone propionate. Methyltestosterone at 8.0 mg./egg produced slightly greater inhibition of HAd 1 than did testosterone propionate at 20.0 mg./egg.

TABLE 4

EFFECT OF TESTOSTERONE PROPIONATE ON HUMAN TUMORS IN EGGS*

Tumor	Mortality		Av. embryo wt. (gm.)		Av. tumor wt. (mg.)		Results (% inhib.)	
	C	T	C	T	C	T	Embryo	Tumor
A 42.	1/6	2/8	17	17	1187	637		46
HAd 1.	0/8	1/6	22	19	429	301	14	30
HS 1.	2/7	3/7	22	20	647	285	9	56

* Testing was done with a dosage of 20.0 mg./egg.

Summary

Hormones and related substances are being tested against 4 types of transplantable human tumors, HEp 3, HAd 1, HS 1, and A 42, growing on the CAM of chick embryos. The general procedures used in these tests have been indicated.

Some of the preliminary results have been presented to illustrate the types of responses encountered when hormones and related substances are tested against transplantable human tumors grown in the embryonated egg. These preliminary studies have given indications that human tumors growing on the CAM of chick embryos may be useful in the evaluation of potential antitumor agents among hormones and related substances.

Since steroids are commonly used as conditioning agents to grow human tumors in other hosts, the chick embryo, which allows human tumor growth without conditioning agents, may provide a unique way of testing steroids and related agents against human tumors outside the patient.

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Part VI. The Human Tumor in Heterologous Hosts

THE TRANSPLANTABLE HUMAN TUMOR*

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Early in this century Murphy[1, 2] first announced his theory that resistance of a host animal to grafts of tissues other than its own was derived from the lymphoid cells. On the basis of this concept, he X-irradiated rats in order to destroy their lymphocytes and subsequently implanted mouse tumors in them. Some of these grew but, since none became permanently transplantable, the method was regarded as a type of legerdemain and dismissed as offering little of practical value. It should be noted that Murphy, as well as a number of investigators who followed him, considered the lymphocyte not as part of an over-all immune response but rather as a ready and immediate defense on the part of the host.

Shortly after Medawar[3] related the rejection of homologous skin grafts by a host animal to an immune response, interest again centered on the lymphoid cell, which occurred in such great numbers around the sloughing foreign tissue. These cells were also found around regressing transplantable murine tumors, such as the 6C3HED lymphosarcoma implanted in A mice, and it was learned by us, applying Murphy's early findings, that prior X irradiation of the A host made it so susceptible to the C3H tumors that the latter grew progressively until the death of the A mouse. By extension of this technique it was found that even human tissues, both normal and malignant, could be grown and transplanted successfully in heterologous hosts properly treated with X irradiation.[4, 5] However, to attain optimal conditioning of these animal hosts, a long and often disheartening period of search, trial, and error was found necessary.

We eventually observed, not only that animal species vary in their sensitivity to X irradiation, but also that the different strains respond differently to the same dosage of X rays. Of 6 common strains of rats tested, for example, the Wistar rat was the most sensitive to radiation and the Sprague-Dawley the least. Obviously, an investigator who employs the latter rat and uses the amount of radiation for conditioning recommended for the Wistar animal will be disappointed in his human tissue implantations. We found that the age of the host is also important. The weanling rat, which weighs approximately 50 to 60 gm., was more affected by radiation than adults of any age, or even nurslings. In agreement with this finding is Kaplan's observation[6] that 2 different strains of mice, the A and the C57 Black, X-irradiated at 1 month, were more likely to develop leukemia in later life than animals 2 weeks younger or 1 month older, an indication that lymphoid cells may be especially

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sensitive at this time. Abrams[7] had also found that 30-day-old weanling mice were more susceptible to radiation than animals either older or younger.

Early work with transplantable human tumors and normal or embryonic tissues was thus done with weanling Wistar rats X irradiated from 1 to 6 days prior to implantation. When cortisone* became available, it was found that this drug acts synergistically with the X irradiation, so prolonging its effect that tissues that would otherwise regress after 10 to 14 days, when the inhibition imposed by the radiation disappears, can now be maintained indefinitely.[8, 9] Comparison of the relative efficacy of conditioning with X ray alone, with radiation plus cortisone, or with cortisone alone indicated that for the rat a combination of the two is by far the most effective. For the hamster, and also for the rabbit, however, cortisone alone is preferable. Mice can be treated with both agents, or with cortisone alone.[10] The advent of this adrenal steroid in readily available market quantities has made possible the transplantable human tumors we have today, as well as normal and embryonic tissues such as gut and stomach,[11-13] which can be used for testing purposes while they are proliferating and functioning in or on homologous or heterologous hosts.

At the present time conditioning with X irradiation and/or cortisone no longer seems to produce the simple 1:1 correlation that it once did; that is, an estimate of the effects of conditioning on the lymphoid cell does not provide a criterion for directly evaluating the host's susceptibility to grafting. The action of the two agents is probably not merely additive, and their effects are certainly not identical. It is necessary, for example, to irradiate the rat prior to implantation of foreign tissue. This view is supported by the findings of Taliaferro[14, 15] and others that, to inhibit the action of an immune system, radiation must be given before the antigen for, once the immune mechanism has been set in motion, the remainder of the system is relatively radioresistant. On the other hand, pretreatment with cortisone, especially in grafting procedures, can be exeedingly unwise; the host connective tissue may become inert, preventing the graft from adhering for the simple reason that no stroma or vascularization has been provided.[13] This, of course, has nothing to do with the immune response, but it must be borne in mind.

In any conditioning procedure employing adrenal steroids one must also consider what the pattern of the host's normal steroid excretion may be. Whereas an ACTH-excreting tumor, such as that in mice described by Furth elsewhere in this monograph, can promote the growth of human tumors in these animals, probably by acting through the adrenals, ACTH administered to a rabbit will cause excretion of corticosterone or B, its normal steroid, which will not prolong graft survival in the amount produced.

It is important to the investigator who wishes to use human neoplasms for chemotherapy and other studies that the neoplasms have remained human in both their chromosome[16] and antigen[17] composition, even though some of them have now been transplanted for as long as six years. Indeed, no loss of antigen has been noted during this long period, nor have any of the tumors in our laboratory ever grown in hosts without the same amount of conditioning originally required. We believe that this is a further indication of their stability. This stability is especially noteworthy because some tumors proliferate so rapidly from the time of their first implantation that an initial injection of 200 to 500 mg. of tissue will grow to a size of 10 gm. or more within 11 to 13 days and kill the host, although only an initial and minimal conditioning regime has been used (FIGURE 1). Such tumors represent a volume of growing cells requiring only an initial radiation or cortisone treatment of the host to gain a "foothold." In the ensuing conflict between whatever immunological forces the host can muster and the proliferating tumor, the latter completely outstrips anything the host can offer. There are very few neoplasms of this type. Certainly they do not grow in this fashion even in the donor patients, who would die in a few days if they did. By far the greatest percentage of transplanted human tumors requires a steady continued conditioning of the host in order to grow progressively.

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* About one third of the cortisone acetate used for these experiments was supplied as Cortone by Merck & Co., Inc., Rahway, N. J.

Once human tumors have been grown in the conditioned laboratory animal, they will proliferate also in the embryonated egg[18-21] or tissue culture.[22] This is of interest, since no original human tumors have yet been grown on the chorioallantois of the egg with any degree of success; moreover, in tissue culture only relatively few, although certainly some, have been propagated. A comparative study of implantations in animals, eggs, and tissue culture of the same original human tumors fresh from the operating room has shown that the conditioned animal host is by far the best medium for starting a tumor growth. It may be that animal passage removes some inhibitory factor such as stroma from the human host of origin, as has been suggested elsewhere.[23] As one would expect, the rate of growth of any one neoplasm is not always the same in animals, tissue culture, and eggs. HEp 3 is an extremely virulent tumor in the animal and in the egg, where it metastasizes widely, yet in tissue culture it is somewhat "delicate." On the other hand, HEp 1 or HEp 2, both of which proliferate relatively slowly in the conditioned rat or hamster or on the egg, grow in gallon lots in tissue culture; when returned to the animal host they return to their former indolent pace. Even in the same host the tumor may grow better in one site than in another. For example, HEp 1 does especially well when implanted intraperitoneally (FIGURES 2, 3, and 4). Obviously, the variation in satisfaction of nutritional requirements alone could account for the differences described. In this respect, transplantable human tumors do not differ from the well-known rodent cancers. It is important that human tumors can be transferred with impunity from rats to eggs to tissue culture and back again to animals without any loss of identity with respect to morphology, chromosome make-up, and the quality and quantity of human antigens.

The practical use of human cancers for various studies[18, 19, 24-29] and the relative values of the various systems, for example, animal, egg, and tissue culture, are described elsewhere in this monograph. One factor should be noted that is important to those who wish to work intermittently with quantities of these materials in chemotherapy experiments, but do not want to maintain large numbers of stock tumor-bearing animals at all times: all

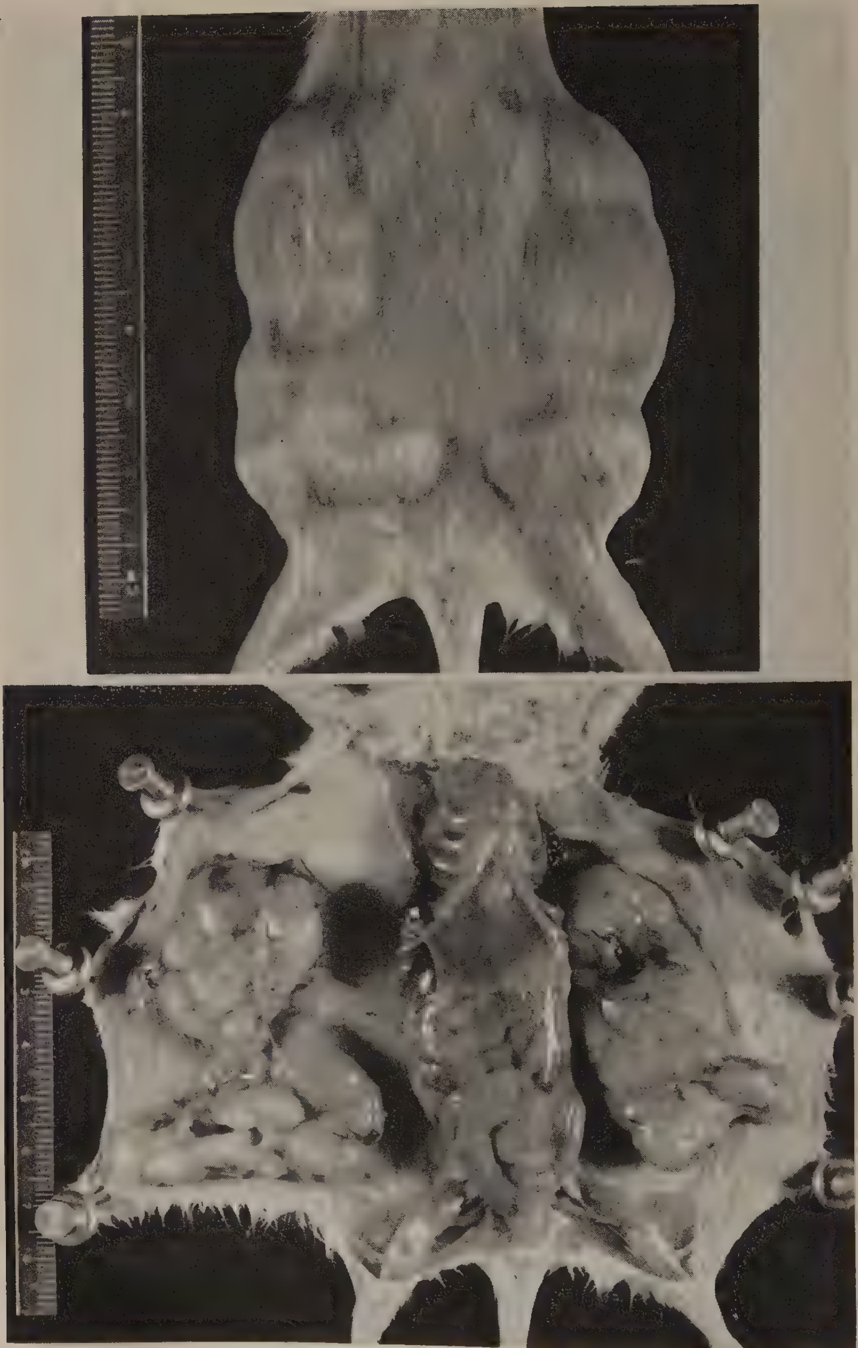

FIGURE 1. The transplantable human tumor HS 1 (human sarcoma No. 1) in its one hundred and twenty-sixth generation 12 days after implantation in both flanks of an X-irradiated rat given 2 doses of cortisone of 3 mg. each. Such a rapidly growing neoplasm kills the host in 11 to 14 days.

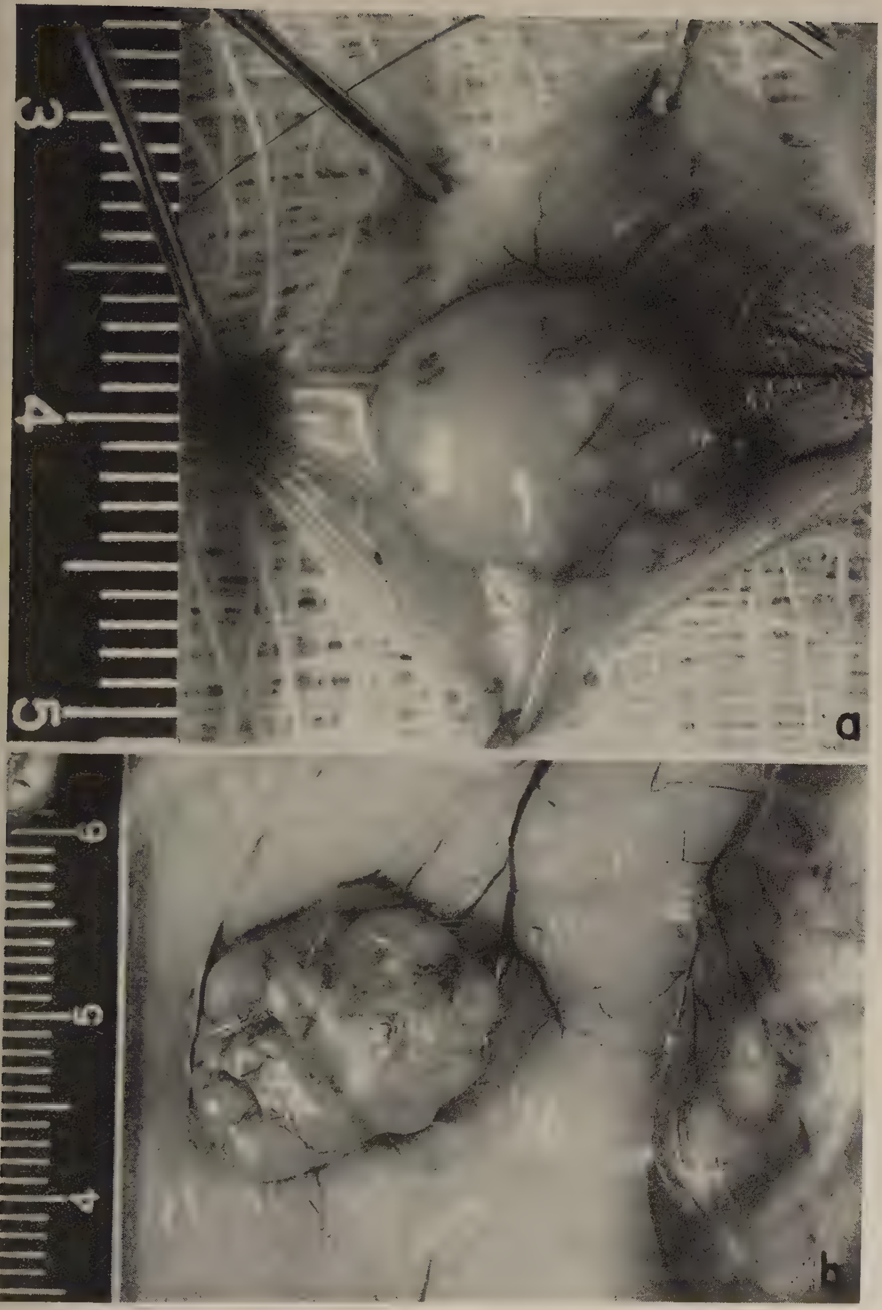

FIGURE 2. Comparison of the growth of the seventy-seventh generation of HEp 1, equal volumes of which were implanted 14 days previously in (a) the hamster pouch, (b) the rat flank, and the hamster peritoneal cavity (see FIGURE 3). All animals were conditioned. The peritoneal cavity produces the greatest yield of this tumor in animal hosts, where it grows more slowly than HEp 3, HEp 5, or HS 1. However, HEp 1 proliferates in tissue culture far more rapidly than these other 3 neoplasms do.

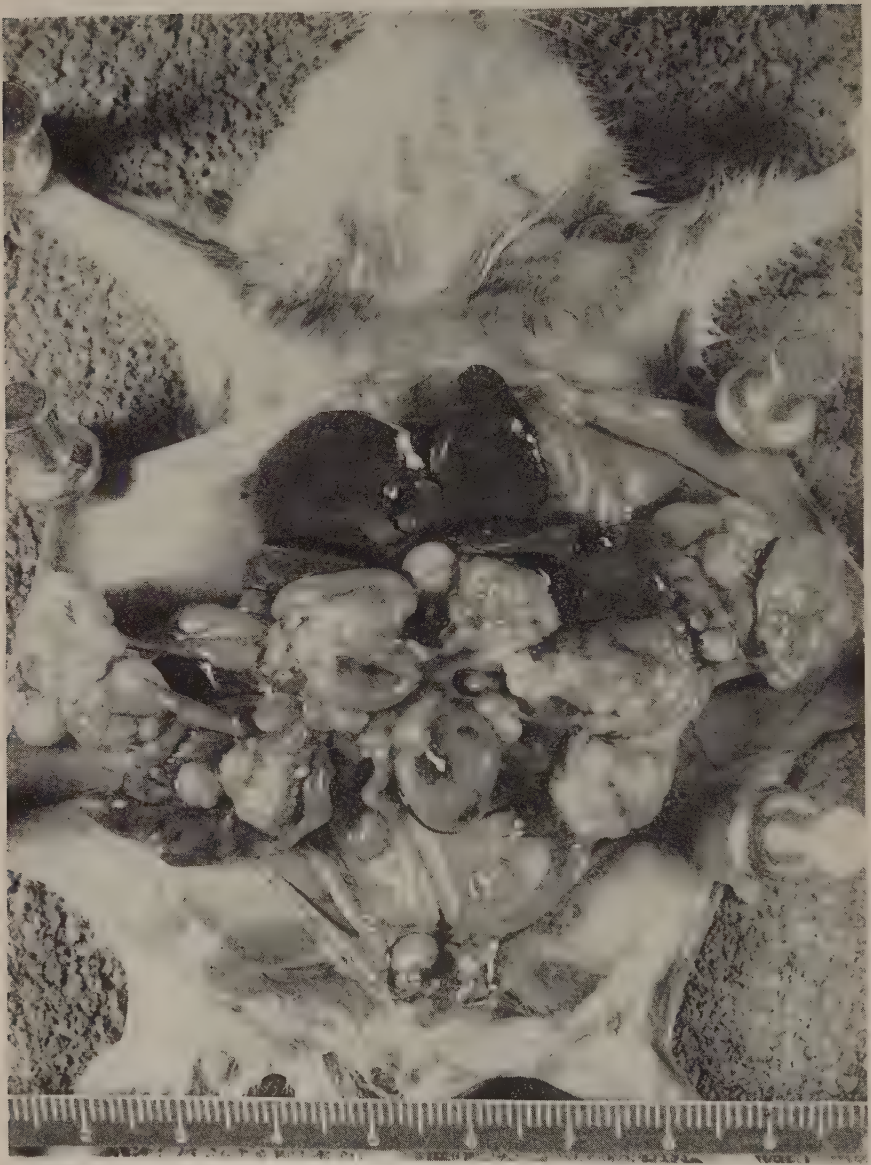

FIGURE 3. Growth of the seventy-seventh generation of HEp 1 in the hamster peritoneal cavity. Compare with FIGURES 2*a* and *b*.

the transplantable human neoplasms thus far tested can be frozen at −75° C. in a dry ice chest and later recovered after thawing at 37° C. and then implanting into conditioned hosts.[30] Six months is the longest freezing period yet tested. The different tumors vary in the ease with which this can be done, as well as in the method best suited to each. Moreover, they grow somewhat unevenly in the first generation. In the second generation, however, uniform growth in all animals implanted is once more established. No loss of specific antigens has been found after freezing.

Descriptions of the morphology of the transplantable human tumors now available have also been given in detail elsewhere. They vary in type from a group of epidermoid carcinomas which, in general, are the easiest to grow, to various kinds of sarcomas, melanomas, adenocarcinomas of the bowel, and even functionally active and secreting testicular tumors.[8, 9, 31-34] Indeed, almost every type of neoplasm is represented for, as the number of investigators interested in obtaining neoplasms of a specific kind has increased, so has the variety of permanently transplantable human tumors. It seems that almost any cancer can be grown in the conditioned host, provided the special needs of that particular growth are understood. Adenocarcinomas of the bowel, for example, must be put into the animal host within minutes after removal from the patient, possibly because of autolytic enzymes. Consequently, it is important to comprehend that transplantability may be related to many factors, among which are the constitution and needs of the tumor, the suitability of the site and host supplied and the relative health of both, and the efficacy of the conditioning procedure, which involves a thorough consideration of whatever side effects it may have.

In sum, then, transplantable human tumors are a reality. In rats and mice they are usually grown with the help of X-irradiation and cortisone conditioning, in hamsters with the aid of cortisone alone, and in eggs and tissue culture without treatment. Their final value for experimental chemotherapy can be assessed only by future events. Certainly they seem to provide a new tool for the study of cancer problems and thus, to a certain extent, they bridge the great gap between studies on murine neoplasms and on the cancer patient.

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Discussion of the Paper

QUESTION: In how many animals were tumor metastases seen in the hamster cheek pouch technique?

TOOLAN: We have seen metastases only from HEp 3 in the hamster cheek pouch. HEp 3 also metastasizes rapidly and widely in the rat and in the chicken egg. Both HEp 1 and HEp 2 have metastasized in the rat.

THE HUMAN TUMOR IN CANCER CHEMOTHERAPY IN THE CONDITIONED RAT*

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Introduction

A great variety of tumor and nontumor systems is being used in the screening of chemical compounds for potential anticancer activity.[1] Those systems involving *in vivo* testing rely for the most part on transplantable animal tumors, which are now available in large numbers and types and can be used with relative ease.[2] These animal neoplasms have been used for studies impossible to make directly on tumors in man.

Recently, both transplantable human neoplasms and suitable methods for growing them have been under investigation.[3] It was believed that such tumors might retain "human" characteristics that could make them uniquely useful in the search for chemotherapeutic agents. With the origination of a method for transplanting and growing tumors of human origin in large numbers of laboratory animals,[4] a new tool for *in vivo* testing of potential anticancer agents has become available.

The use of human tumors in experimental chemotherapy in tissue culture,[5-9] in embryonated eggs,[10, 11] in hamsters,[12] and in rats,[13-20] has been reported. The studies reported below were carried out to develop methods for testing such chemical compounds against human tumors growing in the rat.

Materials and Methods

Toolan's conditioning method was used, with slight modifications. For convenience, a brief outline of the method follows. Young female albino Wistar rats† approximately 4 weeks old were X irradiated with a single total body dose of 150 r between 1 and 4 days prior to transplantation of human tumor tissue. Immediately after transplantation, the rats were injected subcutaneously near the nape of the neck with 60 mg./kg. cortisone acetate,‡ and this was done again on alternate days for a total of 4 injections. In some experiments 3 injections of cortisone at intervals of 3 or 4 days were given to HEp 3-bearing rats.

The 2 human tumors used in these studies were the epidermoid carcinoma of buccal origin, HEp 3, and a sarcoma originating in the soft part of the leg, HS 1, which is described elsewhere.[4] Inoculum for transplantation was prepared in the following manner. Tumors growing subcutaneously in conditioned rats were excised, minced with scalpels, and prepared as a suspension in Locke-

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† Wistar rats were obtained from Carworth Farms, New City, N. Y., and The Charles River Breeding Laboratories, North Wilmington, Mass.

‡ Cortisone acetate, Cortone, was supplied by Merck Sharp & Dohme, West Point, Pa.

Ringer's salt solution. Each 100 ml. of this solution contained 20,000 units penicillin, 60 mg. streptomycin, 600 mg. glucose, and 1 ml. McIlvane's phosphate-citrate buffer at *p*H 7.4. A sample of tumor tissue was removed for a sterility test prior to addition of the diluent.

For experimental chemotherapy, inoculum was prepared from 9- to 11-day-old HEp 3 or HS 1 tumors. Approximately 300 mg. of tissue in one half ml. of suspension was injected subcutaneously into the right flank of X-irradiated rats weighing 55 to 63 gm. Rats transplanted with the same tissue were divided into groups of 5, 6, or 8 per treatment, and a similar number was used as control. Where more than one tumor was used, rats transplanted with the same tumors were distributed equally among each of the treated and control groups. Therapy was started 24 hours after the tumor transplantation and continued as single, daily intraperitoneal injections, with the exception of Sundays, for 9 injections for HS 1 and 7 to 8 injections for HEp 3 tumor-bearing rats. Solutions in physiological saline and suspensions in 0.5 per cent carboxymethylcellulose in physiological saline were prepared in such concentrations that the dose administered was in 0.5 to 0.6 ml. Where necessary, these were prepared daily. Control animals were injected with 0.5 ml. of the diluent. The tests were run at tolerated doses, at least 2 tests being made at each dose. The animals were sacrificed on the ninth to the eleventh day for HEp 3 tumors and on the eleventh to the twelfth day for HS 1 tumors between 24 and 48 hours after the last dose had been given. The tumors were excised, opened, and the debris was removed before they were weighed. Only those tumors weighing 0.3 gm. or more were considered takes.

The effects of conditioning on nontumor-bearing rats were determined on groups of 5 rats per treatment. The treatments were X radiation, a single total body dose being 150 r; cortisone, 1 subcutaneous injection on each of 4 alternate days; X radiation plus cortisone; and no treatment for controls.

All instruments and glassware were sterilized, and aseptic conditions were maintained throughout by the use of glass-enclosed hoods. Purina Lab Chow, supplemented with rolled oats and sunflower seeds, and water were fed ad libitum. The animal room was maintained at approximately 74° F.

Results

The effects of conditioning on the growth of young female, nontumor-bearing rats were determined. Average weight gains over a period of 14 days are given in FIGURE 1.

Untreated rats and X-irradiated rats gained weight rapidly, reaching 124 to 128 gm. by the eleventh day. It was possible to fit a single curve to the data on weight gain for both groups. Animals treated with cortisone, with or without X radiation, gained little or no weight before the eighth day; the last cortisone dose was given on the sixth day. Thereafter, these rats gained in weight, averaging 73 to 76 gm. on the eleventh day. Here, too, one curve fits the data for both groups.

Host mortality, tumor takes, and weight data for a 12-month period are presented in TABLE 1.

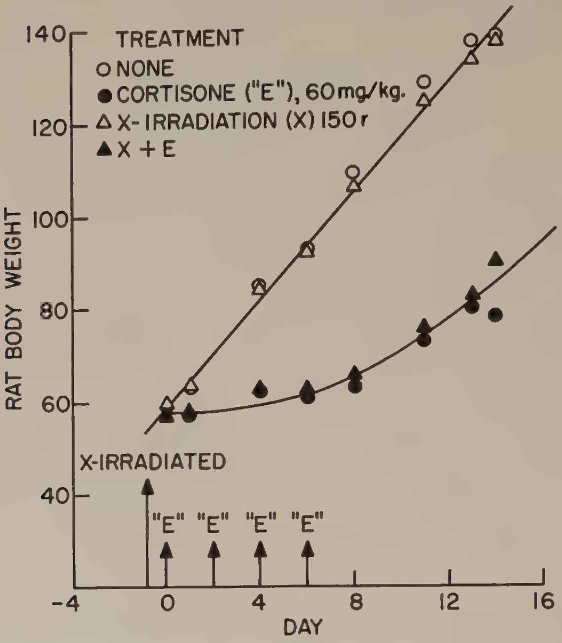

FIGURE 1. Effect of conditioning on gain in body weight of nontumor-bearing, young female Wistar rats.

TABLE 1
POPULATION DATA DERIVED FROM HEp 3 AND HS 1 TUMOR-BEARING RATS FOR A 12-MONTH PERIOD

	HEp 3	HS 1
Number of rats transplanted	726	512
Takes (percentage)	99	88
Mortality (percentage)	9	11
Mean tumor weight (gm.)	3.33	4.95
Standard deviation (gm.)	1.75	3.60
Coefficient of variation (percentage)	52.6	72.7

The take for 726 rats transplanted with HEp 3 was 99 per cent; host mortality was 9 per cent. Tumor weights averaged 3.33 gm. with a standard deviation of 1.75 and a coefficient of variation of 52.6 per cent. The take for 512 rats transplanted with HS 1 was 88 per cent, and host mortality averaged 11 per cent. The mean weight was 4.95 gm. with a standard deviation of 3.60 and a coefficient of variation of 72.7 per cent.

Average tumor weights for each month for a period of 12 months are given in FIGURES 2 and 3. The smallest HEp 3 tumor weights (FIGURE 2) occurred in the months of January and February. There was a sudden increase in tumor size in August, followed in the next 2 months by a return to the area near the grand mean.

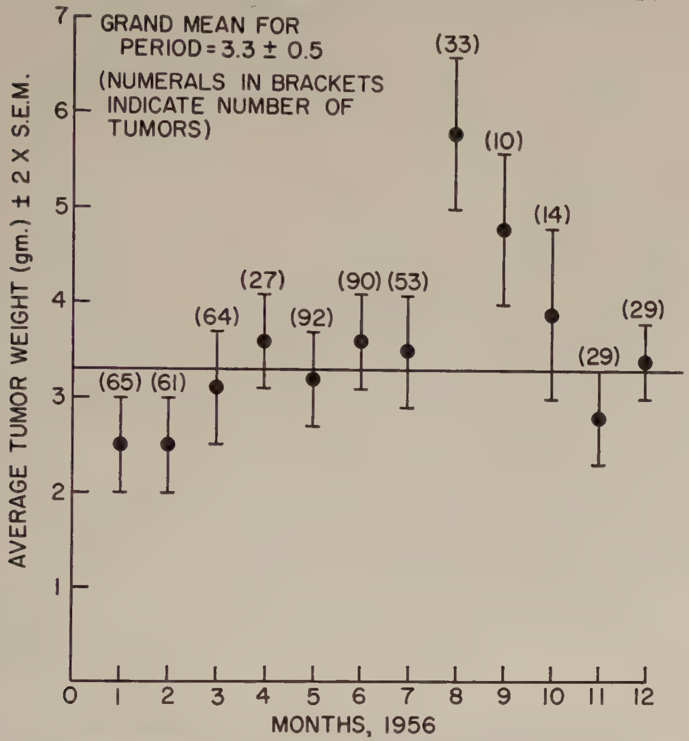

FIGURE 2. Average weight [±2 × Standard Error of the Mean (S.E.M.)] per month of HEp 3 tumors for the period from January through December, 1956.

HS 1 tumors grew at different rates during January, October, and December, the largest tumors occurring in January, and the smallest in the latter months (FIGURE 3). In general, HS 1 tumors grew larger and with greater variability than did HEp 3 tumors.

The results of tests with several chemical compounds are given in TABLES 2 and 3.

Slight or no inhibition of either tumor occurred with A-Methopterin, 5-bromouridine, methionine sulfoxamine, and 6-diazo-5-oxonorleucine (DON). HEp 3 was not inhibited by 5-hydroxyuridine, while inhibition of HS 1 was borderline (25 to 40 per cent). Desacetylthiocolchicine inhibited HS 1, but had questionable activity against HEp 3 because of toxicity associated with this dose. Both tumors were inhibited moderately by actinomycin D and, to a greater degree, by fumagillin.

Discussion

The problem under consideration is the adaptation of transplantable human tumors for experimental chemotherapy studies, and the initiation and carrying on of such studies.

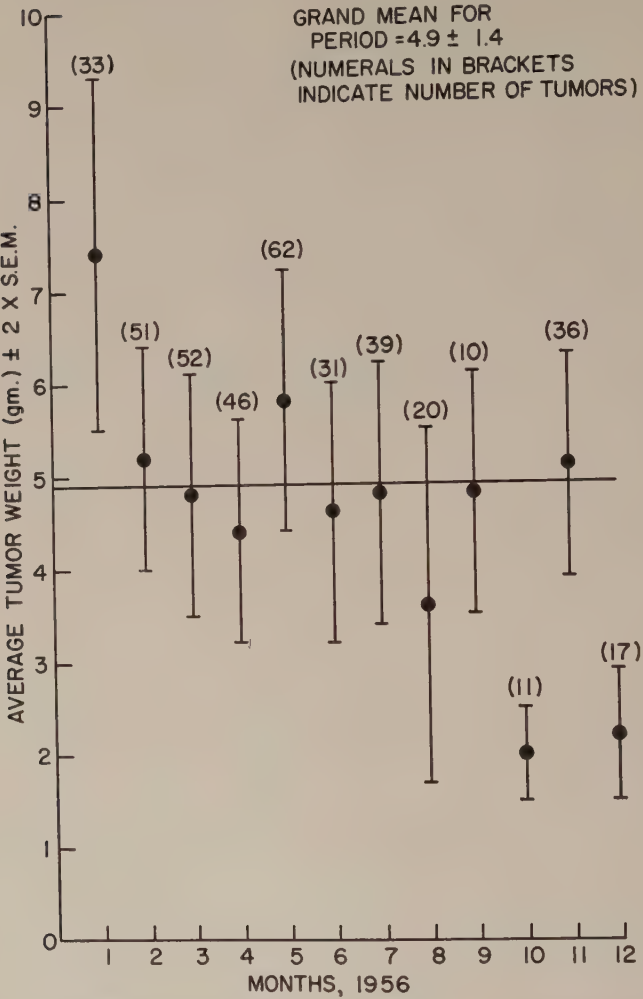

FIGURE 3. Average weight (±2 × S.E.M.) per month of HS 1 tumors for the period from January through December, 1956.

The epidermoid carcinoma HEp 3 and the sarcoma HS 1 were selected because of their rapid rates of growth; these tumors sometimes weigh as much as 15 to 30 gm. in 14 days from 800 mg. of inoculum. To avoid burdening the young rat with too large and unwieldy a growth, an inoculum size of approximately 300 mg. was selected. The resulting tumor growth was of such size, then, that the host was not overwhelmed, and the latent period of growth was reasonably short. Using this implant size, HS 1 grew progressively in conditioned rats for at least 14 days, and HEp 3 for at least 11 days before regressive changes began to appear. Despite the appearance of these changes, tumors increased in size until at least the seventeenth day.[20] Consequently, experi-

TABLE 2

EFFECTS OF SEVERAL POTENTIAL ANTITUMOR COMPOUNDS ON THE GROWTH OF HEp 3 IN RATS

Compound	Dose (mg./kg.)	Toxicity				Inhibition (percentage)
		Av. body wt. Δ		Mortality		
		T (gm.)	C (gm.)	T	C	
A-Methopterin	0.2	+5	+8	0/7	0/8	15
		−4	−3	0/6	0/6	0
		−1	+4	1/6	0/6	6
		+5	+7	0/5	0/5	11
5-Bromouridine	500	+5	+6	0/6	0/6	0
		+6	+7	1/5	0/5	0
Fumagillin	6.25	−4	+7	0/8	0/8	76
		−3	+7	0/8	0/8	75
5-Hydroxyuridine	250	−1	+6	2/8	5/8	0
		+1	−3	1/6	0/6	0
Methionine sulfoximine	18	+6	+10	0/4	0/4	35
		+1	+11	0/6	0/6	19
DON	0.05	−4	−8	0/6	1/6	0
		0	+6	0/6	0/6	11
		−6	−2	0/6	0/6	31
Actinomycin D	0.03	+4	+1	1/6	1/6	64
		−8	−8	0/9	2/9	32
		+3	+7	0/8	0/8	38
		−7	−2	0/5	0/5	86
		−5	0	0/5	0/5	43
		−5	+7	0/5	1/5	34
		+4	+5	0/6	1/6	44
Desacetylthiocolchicine	10	−3	+2	1/6	0/6	0
		−2	+3	3/8	0/7	39*
		−12	−1	2/8	1/8	46*

* Accompanied by toxicity: host weight loss of >6 gm. and/or host mortality >1 in 6, when different from that in the controls.

mental groups were terminated on the eleventh or twelfth day after transplantation for HS 1, and between the ninth and eleventh days for HEp 3 tumors.

Rats selected for experimental chemotherapy weighed 55 to 63 gm. because the number of takes in larger animals was found to decrease. Animals weighing below 55 gm. were not used because of their greater susceptibility to drug toxicity.

The evaluation of a tumor-inhibiting effect in experimental chemotherapy takes into account the degree of variability of tumor sizes of the particular tumor used. Coefficients of variation were found to be 52.6 per cent for HEp 3 tumors and 72.7 per cent for HS 1 tumors (TABLE 1). Both the natural and the induced host immunological resistance to the transplanted heterologous tissue may account for at least a part of this variability in tumor sizes. An examination of the month-to-month variation in mean tumor weights (FIGURES 1 and 2) indicates that fluctuations occur. However, although mean weights may vary, the limits (2 × S.E.M.) associated with each month's mean tumor

Table 3

Effects of Several Potential Antitumor Compounds on the Growth of HS 1 in Rats

Compound	Dose (mg./kg.)	Toxicity				Inhibition (percentage)
		Av. body wt. Δ		Mortality		
		T (gm.)	C (gm.)	T	C	
A-Methopterin	0.2	−2	−6	1/8	2/8	0
		+8	+15	0/4	0/4	35
		+5	+6	1/5	0/5	20
		+3	+9	0/6	0/6	21
5-Bromouridine	500	+3	−1	0/5	0/5	0
		+9	+9	1/6	0/6	14
Fumagillin	1.6	+2	−4	1/5	0/5	79
		+9	−1	2/5	0/5	80
5-Hydroxyuridine	250	−3	+3	1/6	0/6	56
		+1	+6	0/5	0/5	34
Methionine sulfoximine	18	+5	+12	0/6	0/6	41
		+7	+18	0/6	0/6	13
DON	0.025	+3	+4	2/8	3/8	22
		−1	+5	2/8	2/8	36
		+7	+13	0/5	1/5	16
Actinomycin D	0.03	+3	+5	4/12	0/12	26*
		−11	−2	2/5	0/5	50*
		−4	+9	0/5	0/5	78
		+10	+4	0/5	0/5	41
		−1	+13	1/8	4/8	65
Desacetylthiocolchicine	10	−1	+2	4/12	3/12	82
		−5	0	4/8	2/8	46
		+16	+18	3/10	3/10	77

* Accompanied by toxicity: host weight loss > 6 gm. and/or host mortality > 1 in 6, when different from that in the controls.

weight tend to overlap. So-called permanent and transient seasonal tumor variations have been described,[21] and Walpole[22] found that the Walker 256 carcinoma varied considerably in size, although conditions had been standardized as far as was practicable. The marked rise in HEp 3 tumor weights during the month of August and the large tumor weights during the next month are not believed to be seasonal. This phenomenon was not observed with HS 1 tumors, nor did there appear to be a definite pattern in the latter case. In general, month-to-month variations in tumor sizes were not thought to be serious. Attempts are being made, however, to reduce variability in order to reduce the numbers of tests necessary for evaluating a test compound.

Attempts are being made also to uncover alternative conditioning procedures that would allow these tumors to grow without affecting the gain in body weight.[23] Although cortisone inhibits the gain in body weight of the young female rat (FIGURE 1), and a test drug may possibly influence the activity of the conditioning agents, this system offers a unique opportunity to study the effects of potential anticancer compounds on the growth of human tumors in the laboratory.

The number of takes for a 12-month period was higher for HEp 3, 99 per cent, than for HS 1 tumors, 88 per cent. This may be due to the greater growth potential and invasive quality of HEp 3.[4] For shorter periods of time, however, the takes for HS 1 usually average between 95 and 100 per cent. These percentages of takes are comparable with those of transplantable animal tumors. Host mortality, averaging 9 to 11 per cent, was found not to be a deterrent in the evaluation of a test.

Neither tumor was affected by all the compounds tested (TABLES 2 and 3). Since the range of response for each tumor was from 0 to 80 per cent, compounds can be classified in an order of biological activity. Also, chemotherapy data show that the tumors can respond differently to the same agents. This combination of sensitivity to agents, from 0 to 80 per cent inhibition, and differential responses between tumors may add to the usefulness of these particular human tumors for chemotherapy testing.

The effects of the compounds were, for the most part, repeatable. Deviations occurred occasionally, as with other test systems, especially when borderline effects were encountered. Large inhibitions of growth were usually repeatable.

One of the methods that can aid in the analysis of test data is the control-chart procedure.[24] Control charts with upper and lower limits set at the 95 per cent level of confidence were constructed for average tumor weight and range in tumor size. When sizes for control groups fell within the expected limits of variation, experimental data could be examined with confidence. Experimental groups having control tumor weights outside the control limits were considered as possibly representing some abnormal variation in animals, in the procedure, or in the particular tumor suspension used.

In conclusion, it may be stated that the 2 human transplantable tumors, HEp 3 and HS 1, and the procedures outlined for their use are suitable for experimental chemotherapy. Their use may bring about the earlier detection of valuable anticancer drugs for clinical use.

Summary

Data have been presented describing the growth and other characteristics of 2 human transplantable tumors, HEp 3 and HS 1, in the X-irradiated and cortisone-treated young female Wistar rat. The rapid rates of growth observed permitted termination of experiments at 9 to 12 days after transplantation. Takes averaged 99 per cent for HEp 3 and 88 per cent for HS 1, and host mortality averaged from 9 to 11 per cent. Coefficients of variation for tumor weights averaged 52.6 per cent for HEp 3 and 72.7 per cent for HS 1 tumors. Tests with several chemical compounds indicated that the 2 tumors were differentially inhibited and the range of effects was from 0 to 80 per cent, approximately, for both tumors.

Cortisone, but not X radiation, was found to affect the growth of the non-tumor-bearing host. The representative data affirmed the suitability of these two tumor-host systems for use in experimental chemotherapy.

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We are also indebted to the following for supplies of chemical compounds: G. B. Brown, A. Bendich, and J. Fox, Sloan-Kettering Institute, for 5-bromouridine; D. Visser, University of Southern California Medical School, Los Angeles, Calif., for 5-hydroxyuridine; Merck & Co., Inc., Philadelphia, Pa., for actinomycin D; Parke, Davis & Company, Detroit, Mich., for DON.

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OBSERVATIONS ON CHEMOTHERAPY OF HUMAN TUMORS HS 1 AND HEp 3 IN THE HETEROLOGOUS HOST

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In June of 1954 we obtained from Helene Wallace Toolan of the Sloan-Kettering Institute for Cancer Research, New York, N. Y., two transplantable human neoplasms, a sarcoma (HS 1) and an epidermoid carcinoma (HEp 3). These and other human tumors that can be grown in heterologous hosts have provided experimenters with an entirely new approach to screening drugs for their carcinostatic effect.[1-3] Human in origin, these tumors may be capable of identifying the activity of drugs of specific value against human tumor in man or of detecting the carcinostatic properties of drugs impossible to find on the animal tumor screens.[4-7] We have observed, for example, that Acti-dione in antibiotic broth concentration causes inhibition in the growth of HS 1 in rats. The same broth concentration shows no activity against Sarcoma 180 in mice. A broth containing 0.1 mg. Acti-dione/cc. (1 mg./kg. rat) causes inhibition of HS 1; concentrations of 1 mg./cc. (50 mg./kg. mouse) are necessary to affect the growth of Sarcoma 180.[8] Sugiura and his co-workers have reported Acti-dione to be active at 1.0 mg./kg. against the Murphy-Sturm lymphosarcoma in rats, whereas considerably higher doses were required to inhibit the Crocker sarcoma 180 in mice.[9]

Objections have been raised to the use of human tumor growing in the heterologous host as a test method.[10] For one thing, animals must be conditioned with X irradiation prior to implant and must be given injections of cortisone during the first week of tumor growth. Another objection is the fact that, although tumor grows in a high percentage of animals in which it is implanted, there is considerable variation in the size of the tumors. HS 1 grows in 98 per cent of the rats in which it is implanted.

Figure 1 shows the variation in tumor weights in 265 control rats implanted with HS 1. The average tumor weight is 7.4 gm. HEp 3 grows in 100 per cent of the rats in which it is implanted. Figure 2 shows the distribution of tumor weight in 111 HEp 3 control rats. The average tumor weight is 2.9 gm.

The animal itself, its age and weight, the amount of conditioning it receives, all may contribute to variation. However, we have observed that the main source of variation lies in the specific donor tumor with which the animals are implanted. In the S 180 tumor screen for a particular experiment in which a number of drugs are tested, all the animals can be implanted from a single donor mouse. The human tumor is transplanted by the injection of a suspension of minced tumor. Not enough tumor can be obtained from one donor rat to implant all the animals for a chemotherapy experiment. Several donors are needed. Tumors grown from one donor source may be considerably larger or smaller than the tumors grown from other donor sources. In chemotherapy experiments, we have controlled the problem of variation due to source by implanting and treating the animals according to a randomized block design and by using the appropriate analysis of variance.[11, 12]

After preliminary study of three possible human tumor hosts, we selected the rat as the most suitable for large-scale drug screening.

The duration of active tumor growth for HS 1 implanted in rats is more than 14 days; for HEp 3 it is more than 11 days. Therefore, if HS 1-bearing rats are sacrificed 13 days postimplant and HEp 3-bearing rats are sacrificed 10 days postimplant, inhibiton of tumor growth in treated animals cannot be attributed to plateauing or regression.

Animals implanted with tumor gain weight, but not as normal animals do. This inability of the rat to gain weight normally is due to the conditioning injections of cortisone.

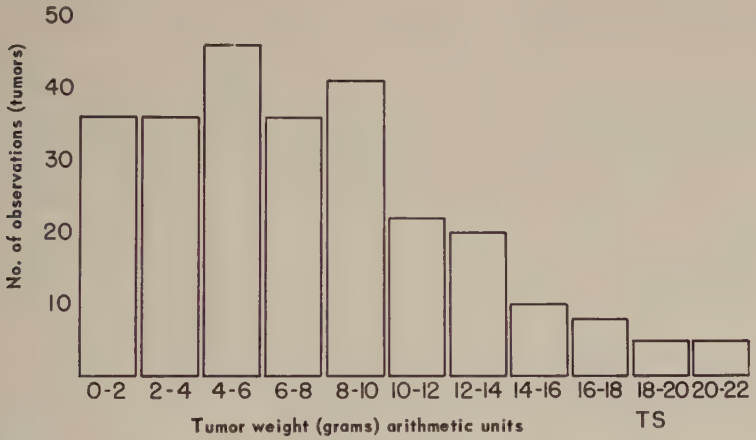

FIGURE 1. Distribution of HS 1 control tumor weights in grams. Control injection, distilled water; number of injections, 12; route of injection, intraperitoneal; duration of growth, 13 days.

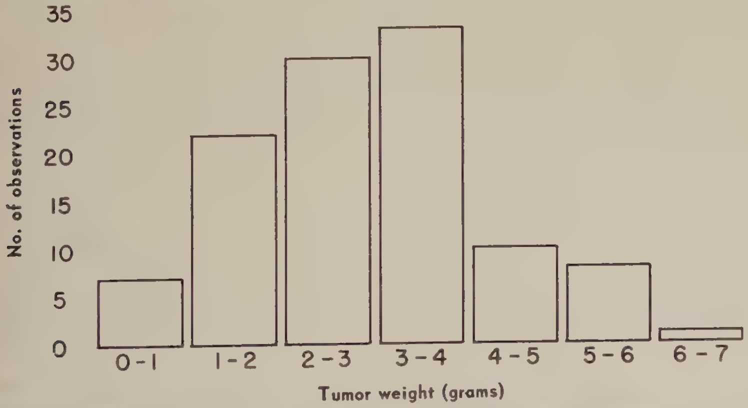

FIGURE 2. Distribution of HEp 3 tumor weights. Duration of tumor growth, 10 days; control treatment, distilled water; route of injection, peritoneal; number of injections, 9.

The mortality rate of control HS 1- and HEp 3-bearing rats is very low; 7.5 per cent of the HS 1 control rats given daily intraperitoneal injections of distilled water die within 13 days. Only 1 per cent of the HEp 3 control rats die within 10 days. Host and tumor should show no reaction to the injection of control treatment. The effect of daily I.P. injections of saline, glucose, and distilled water on host mortality and tumor growth has been compared to the host mortality and tumor growth in rats that receive no injection. FIGURE 3 shows the distribution of percentage of mortality in groups in HS 1 rats injected with one or another of the 3 dummy treatments. Each observation (or experiment) contains 16 rats. Only 1 of the 48 rats that received no injection died. A significantly higher percentage of saline-injected rats died (6 per 16 rats died in 5 experiments; as many as 9, 10, and 11 per 16 rats died in others). Rats injected with glucose showed a higher mortality than rats injected with distilled water (note that in 2 experiments 9 of 16 rats injected with glucose died).

Analysis of the effect of saline, glucose, and distilled water on HS 1 tumor growth showed that saline-injected rats produced tumors significantly smaller than the tumors produced in rats not injected. The weights of tumors in rats injected with glucose or distilled water did not differ significantly from the weights of tumors in rats not injected. Distilled water has, therefore, been accepted as our control treatment.

Our method of transplanting HS 1 and HEp 3 tumors is basically that prescribed by Toolan.

Female albino weanling Wistar rats weighing 40 to 50 gm. and aged approximately 21 to 23 days are considered to be reliable tumor hosts. Rats one week older and weighing between 50 and 60 gm. have shown poorer tumor growth. Age rather than weight may be the critical factor.

Rats are given 150 roentgens X irradiation the day after they are received

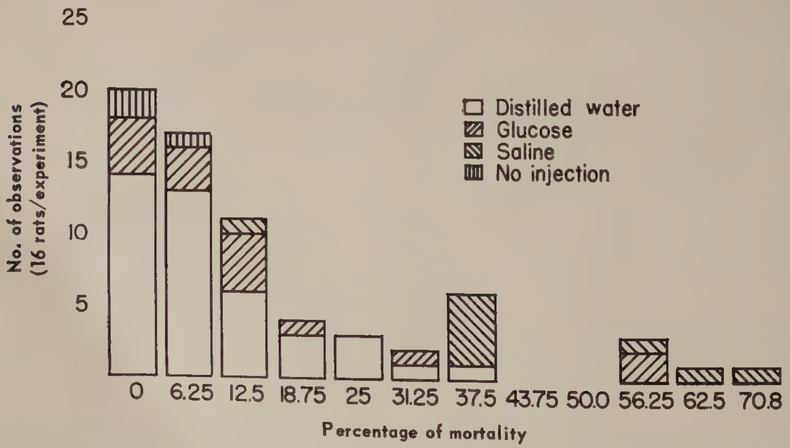

FIGURE 3. Percentage of mortality distribution in HS 1 control rats injected with distilled water, glucose, or saline, and in rats not injected. Duration of tumor growth, 13 days; total number of injections, 12; route of injection, intraperitoneal.

in our laboratory. To assure uniform irradiation, 16 rats at a time are enclosed in a plastic wheel that rotates at 3 rpm for 4½ minutes' exposure. On the morning of the day the rats are to be implanted, each rat receives 3 mg. of hydrocortisone acetate by subcutaneous injection at the nape of the neck. Additional injections of 3 mg. are given on 3 subsequent alternate days.

All rats receive 1 mg. oxytetracycline/cc. drinking water from the day of implant until the day of sacrifice as a protective measure.[13] No adverse effect due to oxytetracycline has been observed on either host or tumor in control or treated rats.

Tumor preparation. HS 1 to be transplanted is selected from rats in which it has grown 12 to 14 days; HEp 3 is selected from rats in which it has grown 9 to 11 days. After the donor host is sacrificed, the tumor is exposed and examined. Only firm vascular tumor, which has a minimum amount of necrotic material, is selected for transplant. Tumor is removed aseptically, cleaned of necrotic material and connective tissue, and bathed in sterile buffered glucose Ringer's solution (BGR) to which have been added potassium penicillin and dihydrostreptomycin. The tumor is minced with scalpels and suspended in BGR. The right side of each conditioned rat is swabbed with alcohol and tumor suspension is injected into the subcutaneous region of the flank. The weight of the rat is taken routinely on the day after implant, 5 or 6 days later, and on the day of sacrifice.

Factorial experiment. Accepting the fact that tumors grown from different host tumors vary in size, there would be no advantage in pooling the rats implanted from several sources and assigning them at random to treatment and control groups. This would interfere with the statistical analysis of the data. The feasibility of pooling the several source tumors before implant was studied.

In the same experiment, animals were injected with either 0.5 cc. or 1 cc. of tumor mince to learn whether the size of inoculum governs the size of tumor growth (TABLE 1). Tumors were removed from 4 donors; the tumors were identical in appearance. Each tumor was divided into equal portions; 1 portion of each was minced and used to implant a group of 6 rats, 3 receiving 0.5 cc. and 3 receiving 1.0 cc. The other halves of the tumors were pooled, minced, and used to implant a group of 24 rats, 12 receiving 0.5 cc. and 12 receiving 1.0 cc. This table shows the difference in the weight of the tumors that developed from tumor C compared with the weight of tumors produced in rats implanted from the other 3 tumor sources.

Tumors grown from 1 cc. of inoculum are not significantly larger than those grown from one half that amount. The pooled tumors are significantly smaller than those grown from the single sources. The one tumor, C, seems to have acted like the proverbial bad apple in the barrel.

Experimental design. Our experiments are designed with consideration of these foregoing facts (D. E. Hutcheon, personal communication). TABLE 2 shows the format for implant. Ten or more compounds are tested; 8 rats are treated with each compound; 2 groups of 8 rats serve as controls. Tumors from 8 single sources are prepared separately and injected 0.5 cc./rat into the number of rats corresponding to the number of treatment and control groups.

TABLE 1

GROWTH OF HS 1 TUMOR FROM FOUR DONORS IMPLANTED SEPARATELY COMPARED WITH GROWTH OF THE SAME FOUR DONORS' TUMORS POOLED

Duration of Tumor Growth: 13 Days

	Tumor A		Tumor B		Tumor C		Tumor D	
Size of inoculum (cc.)	0.5	1.0	0.5	1.0	0.5	1.0	0.5	1.0
Individual tumor weights (gm.)	11.18	7.55	13.03	17.42	3.19	8.65	9.41	12.35
	11.12	16.90	15.10	10.16	2.10	5.94	9.49	9.09
	8.12	13.46	13.56	15.42	3.10	dead	17.76	18.80
Mean tumor weight (gm.)	10.14	12.64	13.90	14.33	2.80	7.30	12.22	13.40

Mean tumor weight of 0.5 cc. implants, 9.76 gm.
Mean tumor weight of 1.0 cc. implants, 12.34 gm.

Tumors A, B, C, and D Pooled Before Implant

Size of inoculum	0.5 cc.		1.0 cc.	
Individual tumor weights (gm.)	5.27	2.98	2.10	dead
	2.75	4.85	dead	7.30
	5.77	8.22	4.00 pm	9.02
	8.83	6.25	4.95	8.49
	1.57 pm	2.23	10.60	6.78
	dead	7.82	4.27	5.82
Mean tumor weight	5.14 gm.		6.33 gm.	

TABLE 2

DESIGN OF IMPLANT FOR CHEMOTHERAPY EXPERIMENTS

Single source tumors	Number of rats implanted	Rat ear mark
1	12	1
2	12	2
3	12	3
4	12	4
5	12	5
6	12	6
7	12	7
8	12	8

Random Separation of Rats into Treatment and Control Groups

Treatment groups	Number of rats/group	Rats ear marks
A Control	8	12345678
B Control	8	12345678
C Treatment	8	12345678
D Treatment	8	12345678
E Treatment	8	12345678
F Treatment	8	12345678
G Treatment	8	12345678
H Treatment	8	12345678
I Treatment	8	12345678
J Treatment	8	12345678
K Treatment	8	12345678
L Treatment	8	12345678

TABLE 3

EFFECT OF DRUGS ON THE GROWTH OF HS 1 IN CONDITIONED RATS

Duration of Tumor Growth: 13 Days
Route of Injection: Intraperitoneal

	Dose mg./kg.	No. of inject.	Ch. in body wt.	Mortal. rate	Tumor take	Mean tumor wt.	Inhib. (%)	Signif.*
Control—distilled water		12	+3.6	1/16	15/15	7.09		
DON	0.02	12	+5.7	0/8	8/8	4.91	31	N.S.
Azaserine	0.25	12	+7.4	0/8	8/8	5.14	27	N.S.
Fumagillin	3	12	+5.5	0/8	8/8	4.20	41	S.
2,6-Diaminopurine	50	10	−6.3	1/8	7/7	2.52	64	S.
6-Mercaptopurine	10	8	−0.2	4/8	4/4	4.42	37	N.S.
Nitrogen mustard	0.2	11	+3.0	1/8	7/7	5.86	17	N.S.
5-Fluorouracil	10	12	+7.7	0/8	8/8	3.51	50	S.
Urethan	300	12	−4.4	0/8	8/8	1.58	78	H.S.
BA 4105	0.2	12	+10.1	1/8	7/7	2.71	62	H.S.

* Symbols: N.S., not significant; S., significant; H.S., highly significant.

The animals implanted from each of the 8 source tumors are earmarked; 1 animal from each source is assigned at random to treatment and control groups, as shown in this table.

Chemotherapy treatment. Treatment by daily intraperitoneal injection of the drugs or with control-distilled water begins 24 hours after implant and terminates 24 hours before the animals are sacrificed. Drugs are diluted so that the animals are injected with approximately 0.5 cc. of the drug per day. HS 1 tumor-bearing rats receive 12 injections, HEp 3-bearing rats receive 9 injections.

When an experiment is terminated, the tumors from the surviving animals are removed, freed of necrotic material and connective tissue, and weighed.

Evaluation of results. The average tumor weight of treated rats is compared with the average tumor weight of the controls. Our statisticians transform the tumor weight to logarithmic units and subject the data to the analysis of variance. The significance of variation is measured by the F test. The significance of the reduction in the tumor weight of each treatment is measured by the one-sided t test.[14]

TABLE 3 shows the reaction of HS 1 tumor in rats to treatment with various known animal tumor inhibitors, as well as to one of our compounds within one experiment. The reduction of tumor growth in rats treated with fumagillin, 2,6-diaminopurine, and 5-fluorouracil is significant at the 5 per cent level. The inhibition caused by urethan and BA 4105 is highly significant. The inhibition indicated for the other compounds is not significant. Nitrogen mustard at nontoxic levels does not show activity.

Both HS 1 and HEp 3 are selective in their reaction to the injection of certain substances. The results are reproducible.

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Discussion of the Paper

Philip C. Merker* (*Division of Human Tumor Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, and Sloan-Kettering Division, Cornell University Medical College, New York, N. Y.*): In addition to theoretical research problems, a screening laboratory is usually faced with making practical day-to-day decisions about the anticancer activity of tested agents. Sometimes these decisions must be based on a limited number of tests conducted with limited amounts of material. When this occurs, it is necessary to interrelate results with test data from other compounds obtained weeks, months, or even years previously. The element of time, when brought into evaluations, can be handled adequately by a statistical device known as a control chart.[1] In fact, the technique of the control chart is concerned solely with variations over time, and therefore has an important place in any screening procedure.

The control chart technique can be described as follows: (1) small samples are taken from past data; (2) limits are established from the pooled deviations, and appropriate charts are constructed for means and deviations; and (3) day-to-day values are plotted into the charts. Control charts for mean tumor weight and range in tumor weight for human sarcoma No. 1 (HS 1) grown subcutaneously in the conditioned young female rat are used to illustrate the application of the control chart procedure.[2]

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FIGURE 1 is a control chart for tumor weight. Data were used from approximately 90 tumors representing approximately 20 control groups accumulated during the 4-month chemotherapy period from April through July, 1957. The grand mean for tumor weights was 5.0 gm., and upper and lower limits set at two sigma—95 per cent confidence level—were computed from the range in tumor weights for sets of 4 tumors. The average tumor weight of each control group used in every chemotherapy test for the months of October, November, and December, 1957 and January, 1958 was plotted on the chart. It can be seen that 3 control groups were "above" the expected limits, and one was "below" the limits set for mean tumor weight. These data show that during this period 86 per cent of the control groups were "in control" for mean tumor weight.

FIGURE 2 shows the variability in tumor weight expressed as range in tumor weight for an average control group consisting of 4 tumors. The base period is the same as that previously described; the average range of 4 tumors was 5.9 gm.; upper and lower limits were set at the 95 per cent level of confidence. Data covering the chemotherapy period indicate that 5 control groups were above the specified limits. Therefore, during this period 86 per cent of the control groups were in control.

Examination of both charts reveals that approximately 80 per cent of all control groups were in control for both mean tumor weight and range. Test groups associated with these control groups could be interrelated over the

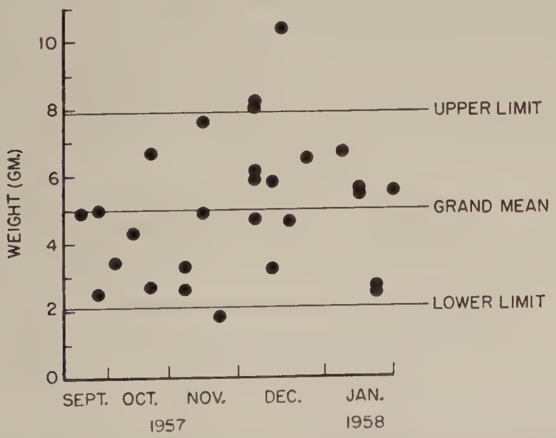

FIGURE 1. Control chart for HS 1 tumor weight in the rat; subcutaneous growth; base period, April 1957 through July 1957; total number of tumors, 93; 2 sigma limits based on 4 tumors.

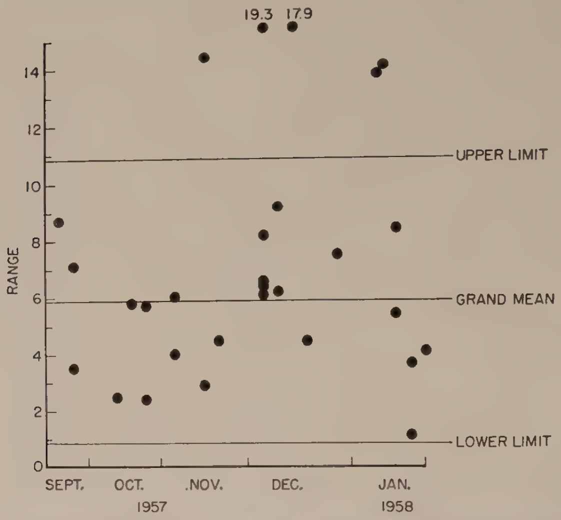

FIGURE 2. Control chart for HS 1 tumor weight range in the rat; subcutaneous growth base period, April 1957 through July 1957; total number of tumors, 93; 2 sigma limits based on 4 tumors.

entire chemotherapy period. Data from test groups associated with out-of-control groups were examined with caution. In this way, the control chart acted as a stop when daily decisions were made about candidate agents.

An additional and equally important function of control charts is to give an objective picture of the general performance of the laboratory. When excessive downward and upward trends are noted there are good reasons for attributing these variations to assignable causes.[3]

Human tumors growing in conditioned animal hosts offer a unique opportunity to experimental cancer chemotherapists. The application of simple visual statistical methods, such as control charts, will facilitate the use of these tumors and further increase the usefulness of chemotherapy data obtained from these transplantable human tumors.

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VERONICA ARMAGHAN AND W. F. DUNNING* (*Cancer Research Laboratory, University of Miami, Coral Gables, Fla.*): Following the demonstration by Helene Wallace Toolan (1954) that HEp 3 and HS 1 could be heterologously transplanted in X-irradiated and cortisone-conditioned rats, an attempt was made to establish these neoplasms in Fischer line 344 rats. The purpose was to determine whether the use of highly inbred hosts would reduce the variability in the growth of the heterologously transplanted neoplasms and thus increase the usefulness of these neoplasms for therapeutic studies.

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* The research for this paper was supported by Grant C-2498 from the National Cancer Institute, Public Health Service, Bethesda, Md.

Fischer line 344 rats were chosen for the study because they were the most easily propagated of the inbred lines of rats available. These rats are highly fertile, relatively small albinos that mature at about 70 days of age; the females wean an average of 6 litters of 8 young each in an average breeding period of 1 year.

They proved to be more resistant to X irradiation than the Wistar rats used by Toolan. Five attempts to establish HEp 3 as a continuously transplantable, progressively growing neoplasm in Fischer line 344 rats were unsuccessful.

The procedure that proved most satisfactory for the growth of HS 1 consisted of 48-hour preimplantation treatment of rats between 28 and 32 days old and weighing 40 to 50 gm. with 250 r of total body radiation, or 3- to 5-hour preimplantation injection of 5 mg. of Cortone Acetate.* Implantation of the tumor was by subcutaneous injection by a hypodermic syringe having a 16-gauge needle of 0.7 to 1.0 ml. of a 50 per cent tumor-cell suspension in a balanced salt solution containing 20,000 units of penicillin per cc. After implantation, four 5-mg. doses of cortone acetate were injected subcutaneously on alternate days.

Tables 1, 2, and 3 summarize the data obtained over a span of 50 transfer generations of HS 1 in the descendants of a single pair of Fischer line 344 rats that had previously been inbred brother by sister for 53 generations. Except for the final group, these data were taken from the inoculated rats that served as controls for therapeutic studies terminated on the fourteenth day after inoculation. The surviving rats were sacrificed, and the neoplastic tissue was excised, separated from the residual necrotic implant, and weighed. The final group consists of rats used in the routine maintenance of the tumor, which were not so accurately weighed, and includes rats that were sacrificed for transplantation on the twelfth to the fourteenth day after inoculation.

Table 1 indicates the number of inoculated rats in each group and the percentage that showed growth of the implanted tissue. In the first group, that is, generations 11 to 16, 70 per cent of the 229 rats inoculated in October through December 1955 were positive. In the following month, generations 17 to 19, 86 per cent of the 259 inoculated rats were positive. Aside from the first group of the study, there was only one other group, generations 48 to 57, inoculated in March through June 1957 that had a significantly higher percentage of inoculated rats in which HS 1 showed growth. Of the total of 1811 rats inoculated in this study, 66 per cent showed growth of the heterologously implanted tissue.

Table 2 shows the minimum, maximum, and mean tumor weight that was excised 14 days after inoculation from the tumor-bearing rats of the same

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* Cortone Acetate was supplied for these experiments by Merck & Co., Inc., Rahway, N. J.

TABLE 1

PERCENTAGE OF POSITIVE FISCHER LINE 344 RATS IN 50 SUCCESSIVE TRANSFER GENERATIONS OF HS 1 AND A COMPARISON OF THE FIRST GROUP (GENERATIONS 11 TO 16) WITH SIX SUBSEQUENT GROUPS

HS 1/gen.	Dates	No. rats inoc.	Percentage positive	Diff. ± P.E.
11–16	Oct.–Dec. 1955	229	69.9	
17–19	Jan. 1956	259	86.5	16.6 ± 2.5
20–23	Feb. 1956	227	76.2	6.3 ± 2.8
24–29	Mar.–June 1956	309	76.7	6.8 ± 2.6
31–41	July 1956–Feb. 1957	231	75.8	5.9 ± 2.8
48–57	Mar.–June 1957	205	86.8	17.0 ± 2.7
58–61	July–Aug. 1957	411	63.0	6.9 ± 2.6

TABLE 2

MINIMUM, MAXIMUM, AND MEAN TUMOR WEIGHT IN GRAMS AT 14 DAYS FOR 50 TRANSFER GENERATIONS OF HS 1 IN FISCHER LINE 344 RATS AND A COMPARISON OF THE FIRST GROUP (GENERATIONS 11 TO 16) WITH SIX SUBSEQUENT GROUPS

HS 1/gen.	Tumors	Tumor weight in grams at 14 days			
		Min.	Max.	Mean ± P.E.	Diff. ± P.E.
11–16	103	0.1	18.0	2.6 ± 0.21	
17–19	182	0.1	19.3	3.6 ± 0.20	1.0 ± 0.29
20–23	148	0.1	16.0	4.0 ± 0.20	1.4 ± 0.29
24–29	206	0.1	17.2	4.0 ± 0.18	1.4 ± 0.28
31–41	129	0.1	17.7	5.7 ± 0.28	3.1 ± 0.35
48–57	174	0.1	19.8	6.6 ± 0.25	4.0 ± 0.33
58–61	259	0.3	10.0	2.3 ± 0.12	−0.3 ± 0.24

TABLE 3

MEANS AND STANDARD DEVIATIONS OF THE TUMOR WEIGHT IN GRAMS AT 14 DAYS FOR 50 TRANSFER GENERATIONS OF HS 1 IN FISCHER LINE 344 RATS AND A COMPARISON OF THE STANDARD DEVIATIONS OF THE FIRST GROUP (GENERATIONS 11 TO 16) WITH SIX SUBSEQUENT GROUPS

HS 1/gen.	Tumors	Tumor weight in grams at 14 days		
		Mean ± P.E.	S.D. ± P.E.	Diff. ± P.E.
11–16	103	2.6 ± 0.21	3.1 ± 0.16	
17–19	182	3.6 ± 0.20	3.9 ± 0.14	0.8 ± 0.21
20–23	148	4.0 ± 0.20	3.6 ± 0.14	0.5 ± 0.21
24–29	206	4.0 ± 0.18	3.9 ± 0.13	0.8 ± 0.21
31–41	129	5.7 ± 0.28	4.7 ± 0.20	1.6 ± 0.26
48–57	174	6.6 ± 0.25	4.9 ± 0.18	1.8 ± 0.24
58–61	259	2.3 ± 0.12	2.8 ± 0.08	−0.3 ± 0.18

groups shown in TABLE 1. TABLE 2 shows that the tumor growth varied from 0.1 to nearly 20 gm., and that from generations 11 through 57 the mean tumor weight increased progressively from 2.6 ± 0.21 to 6.6 ± 0.25 grams, that is, the tumors more than doubled in average weight. For the final group, generations 58 to 61, the average tumor weight was 2.3 ± 0.12 grams, which is not significantly different from the first group.

TABLE 3 shows the standard deviations of the mean tumor weight for the same groups of tumors. In 4 of the 7 groups compared, the standard deviation was equal to or greater than the mean. The variability increased as the average weight of the tumors increased. The cause of the variation remains obscure, which somewhat limits the use of this neoplasm as a tool for exact quantitative studies. It is obvious, however, that the variability was not reduced by successive growth for 50 transfer generations in hosts of the same genetic constitution.

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THE HUMAN TUMOR GROWN IN THE EGG*

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Introduction

Human tumors are grown on the chorioallantoic membrane (CAM) of the chick embryo for chemotherapy studies. The tumors used in these studies are established transplantable human tumors with rapid growth rates. Due to the relatively short time interval between chorioallantoic implantation and evaluation of the results, it is desirable to use tumors with rapid growth rates for chemotherapy studies.

The use of the CAM of the chick embryo for heterologous tumor growth dates back to the classic studies of Murphy (1912). Today the CAM implantation technique for heterologous tumor growth embodies many modifications introduced by various investigators: the removal of a square of shell and the cutting away of the shell membrane to expose the CAM, by Clark (1920); the preference for nine-day-old embryos for tissue grafting and the selection of the point of junction of two or more large blood vessels as a favorable graft site, by Willier (1924); and the creation of an artificial air space above the chorioallantois, by Burnet (1933). The chick embryo has been used to grow tumors originating in a variety of animals (Stevenson, 1918, and Schrek and Avery, 1937), including man (Dagg *et al.*, 1954).

Human tumors grown on the CAM have been shown to respond to certain potential antitumor agents (Dagg *et al.*, 1954 and 1955, and Harris, 1956). The procedures we employ in experimental chemotherapy utilizing human tumors grown on the CAM of the chick embryo are presented below. Experimental results are discussed here only to the extent that they illustrate how this testing method may be utilized. The results presented have a bearing on issues frequently discussed regarding this testing method: the response of different tumor types to a given agent, the response of a given tumor to different agents, the relationship between inhibition of embryo growth and that of tumor growth, and the reproducibility of results.

Procedures

Fertile White Leghorn eggs incubated for nine days are used in our studies. The incubators are maintained at approximately 37° C. The eggs are candled to determine their suitability and to locate a desirable vascularized area, usually one in which a major blood vessel branches in the chorioallantois. The area is indicated on the shell, where the rectangular window is later made.

A high-speed drill with an abrasive disk attachment is used to grind grooves in the shell for the window. Care is exercised to prevent damage to the CAM. An egg punch is used to puncture the shell in the air space area, and the rec-

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tangle of shell is removed from the window area with a pointed instrument. A drop of sterile isotonic saline is placed on the shell membrane, which is then ruptured to permit the saline to pass more freely between the shell and chorioallantoic membranes, thus facilitating the separation of the membranes. Frequently the chorioallantois will "drop," that is, separate from the shell membrane when the latter is ruptured; if this does not occur, gentle suction is applied with a syringe bulb over the hole in the air space. The ruptured shell membrane is removed with forceps, and the eggs are then ready to receive the tumor implants.

The preparation of the tissue includes removing it from its growing site, generally other eggs. The egg is held between the fingers and thumb along the lesser axis and the shell is cut with scissors along the greater axis. The egg is cut in a manner that permits the window to appear in one of the halves, and the tumor is generally found attached to the chorioallantois near the window. Forceps and scissors are used to remove the tumor, which is then placed in a sterile Petri dish and covered with enough sterile isotonic saline to provide moisture. Antibiotics are generally added to the saline as a safeguard against operational contaminants. The tumor is freed of the surrounding membrane, then cut into fragments approximately 3 mm. in size. A single fragment of tumor tissue is placed on the previously selected blood vessels of the CAM. The window is then sealed with transparent tape. Special racks or holders with triangular slots are used to keep the window uppermost for the rest of the experiment.

A period of four days is allowed for the tumors to become established on the CAM; then the eggs are examined by removing the tape from the window and illuminating the implantation site with a diagnostic light assembly. In this manner we are able to select tumor-bearing eggs with a high degree of accuracy and to ensure beginning the experiment with actively growing tumors.

The agent to be tested, if in a powdered or crystalline form, is placed in an appropriate diluent, such as saline, distilled water, or sesame oil. Generally we administer the desired dose of the test agent in volumes of diluent between 0.20 ml. and 0.50 ml. The egg receives a routine single dose of the test agent injected into the yolk sac. Agents are generally tested at the highest dose level tolerated by the embryos. The embryos are sacrificed for evaluations on the nineteenth day of incubation, which is six days after the beginning of therapy. Mean tumor weight comparisons are made between treated and controls, and histological studies are made whenever tumor inhibition is sufficient to warrant them. Similarly, mean embryo weights are compared, and the external and internal features of the treated embryos are examined for gross changes.

These studies are made on Toolan's epidermoid carcinoma (HEp 3), sarcoma (HS 1), adenocarcinoma (HAd 1), and Skiff's bronchiogenic carcinoma (A 42).

Experimental

The effects of a single agent on different tumor types. Certain qualitative and quantitative differences in the response of different tumor types to a given antitumor agent have been observed repeatedly in this test system. Actino-

TABLE 1

EFFECT OF ACTINOMYCIN D ON HUMAN TUMORS IN EGGS

Tumor	Dose (mg./egg)	No. of eggs		Av. tumor wt. (mg.)		Results (% inhib.)
		C	T	C	T	
HS 1	.024	28	25	907	846	− (0–25)
HEp 3	.024	30	29	661	586	− (0–25)
A 42	.01	20	20	1125	384	+ (51–75)
HAd 1	.024	30	14	519	341	± (26–50)

mycin D has been chosen to illustrate the action of agents in this category. It was tested at a dose level of 0.024 mg./egg against HS 1, HEp 3, and HAd 1. For reasons not yet explained, embryos bearing A 42 tumors would not tolerate actinomycin D above 0.01 mg./egg; consequently, the testing against A 42 was limited to this dose level.

TABLE 1 indicates the results of the testing in the form of pooled data from two or more experimental groups per tumor type. It may be noted that actinomycin D had no effect on either HS 1 or on HEp 3, while weak activity was indicated against HAd 1. The most striking feature of the results is the considerably greater activity against A 42, which was tested at less than one half the dose level of the other tumor types.

When the average weights of the various tumor types were compared, it was obvious that the tumors of A 42 were larger than those of the other tumor types. This suggested that A 42 tumors possessed a more rapid growth rate than tumors of the other types, which led us to expect that a growth inhibitor affected the A 42 tumors to a greater extent than it did the others. However, this concept was not supported by a comparison of the average tumor weights of HS 1 and HAd 1, which implied that the tumor type with the lesser growth rate is more responsive. The evidence, then, suggests strongly that A 42 tumors are more sensitive to actinomycin D than are tumors of the other types, as a result of factors other than a greater growth rate. These contrasting responses of tumor types to a common agent present a challenge to determine, biochemically or otherwise, what it is in the nature of these tumor types that accounts for their behavior.

The effect of different agents on a single tumor type. A given type of human tumor grown on the chorioallantois can indicate differences between a series of related antitumor agents. To illustrate this, HEp 3 has been selected as the tumor type and four actinomycins, D, E_1, J, and X_1, have been chosen as test agents. Each of the compounds was tested at its maximum tolerated dose level, which was found to vary among members of the series.

The pooled data from two or more experimental groups per test agent are recorded in TABLE 2. The test agents are identified according to their Sloan-Kettering code numbers as follows: actinomycin D (SK 19095b), actinomycin E_1 (SK 16700), actinomycin J (SK 16701), and actinomycin X_1 (SK 16702). Clearly, HEp 3 did not respond to SK 19095b at 0.024 mg./egg, which is considered a high dose for an actinomycin. The results were also negative in

TABLE 2
EFFECT OF ACTINOMYCINS ON HEP 3 IN EGGS

Compound	Dose (mg./egg)	No. of eggs		Av. tumor wt. (mg.)		Results (% inhib.)
		C	T	C	T	
SK 19095b	.024	30	29	661	586	− (0–25)
SK 16700	.01	14	10	256	163	± (26–50)
SK 16701	.02	12	9	296	107	+ (51–75)
SK 16702	.01	14	10	256	232	− (0–25)

the tests with SK 16702 at the maximum tolerated dose of 0.01 mg./egg. Slight inhibition of HEp 3 was produced by SK 16700 at 0.01 mg./egg, while SK 16701 at 0.02 mg./egg produced moderate inhibition and is considered the most active of the actinomycins in this particular series against HEp 3 in the egg.

The results indicate that a variety of responses may be encountered when using only a single human tumor type as a test object. Beyond the recording of effects lies the challenge to explore the causes.

The relationship between growth inhibition of embryo and of tumors. The results presented here may not be critical proof that inhibition of embryo growth and inhibition of tumor growth are independent of each other, but this is precisely what may be inferred with a high degree of confidence. Thiadiazole was tested against HEp 3 in the egg at 3 dose levels: 10.00 mg./egg, 15.00 mg./egg, and 20.00 mg./egg. The results are recorded in TABLE 3 as a single test at each of the dose levels. The inhibition of the growth of the embryo is comparable to the inhibition of the tumor growth, except at the 10.00-mg./egg dose level. Here the growth of the embryo was inhibited 33 per cent, while the treated tumor weights actually exceeded those of the controls. The results of the thiadiazole tests would not rule out the possibility that the inhibition of embryo growth influenced the inhibition of tumor growth, provided only the 15.00-mg. and 20.00-mg. dose levels were considered, but it is difficult to bring this concept into harmony with the results at the 10.00-mg. dose level. It appears that thiadiazole can inhibit growth, but that it does not act selectively against either normal or neoplastic tissues. It appears also that the responses of normal and of neoplastic tissues to thiadiazole are independent of each other.

TABLE 3
EFFECT OF THIADIAZOLE ON HEP 3 IN EGGS

Test no. and dose (mg./egg)	Mortality		Embryo wt.		Av. tumor wt. (mg.)		Results (% inhib.)	
	C	T	C	T	C	T	Embryo	Tumor
1. 10 mg.	2/8	2/9	21	14	525	651	33	
2. 15 mg.	1/6	2/6	21	14	559	376	33	33
3. 20 mg.	1/6	1/6	21	13	559	328	38	41

Table 4

Effect of Fumagillin on Human Tumors in Eggs*

Test no.	Mortality		Av. embryo wt. (gm.)		Av. tumor wt. (mg.)		Results (% inhib.)	
	C	T	C	T	C	T	Embryo	Tumor†
HEp 3								
1	3/10	3/12	20	14	628	40	30	94
2	0/7	2/7	19	18	608	112	5	82
HS 1								
1	1/8	1/8	20	17	485	97	16	80
2	2/13	3/15	22	17	446	104	23	77

* 2.5 mg./egg.

† $P < 0.01$.

Further indications that inhibition of tumor growth is not dependent on inhibition of embryo growth are shown in TABLE 4. Here fumagillin was tested against HEp 3 and HS 1 at 2.50 mg./egg. There is considerable inhibition of tumor growth, and it is consistent. The effects on the embryo at this dose level were variable, but not strikingly so. In each test the difference between the treated and control tumors was statistically significant. However, in no case did differences between treated and control embryos approach statistical significance.

Reproducibility of results. It has been our observation, with very few exceptions, that highly active compounds or agents will inhibit the growth of a given susceptible tumor to similar degrees on repeated tests. The results are more variable when an agent is only weakly active. Such variability is not particularly striking.

The results of tests using fumagillin at 2.50 mg./egg against HEp 3 and HS 1 (TABLE 4) clearly indicate a high degree of reproducibility in terms of tumor inhibition.

Summary

Four types of transplantable human tumors, HEp 3, HAd 1, HS 1, and A 42, are grown on the CAM of chick embryos and employed as test objects to determine the antitumor activities of potential anticancer agents. The general procedures employed in testing have been outlined.

Experimental results have been presented to illustrate the kinds of responses commonly observed when employing this method of testing. The existence of certain qualitative and quantitative differences between the tumor types in their response to a given test agent is indicated by the results of testing actinomycin D against HS 1, HEp 3, A 42, and HAd 1. The implications of these differences have been discussed. The results obtained when HEp 3 was used to test actinomycins D, E_1, J, and X_1 indicate the capacity of a given tumor type to detect differences between related agents.

Results of testing thiadiazole against HEp 3, and fumagillin against HEp 3 and HS 1 indicates that tumor inhibition is not dependent on the inhibition of embryo growth.

The results of testing thiadiazole in contrast with those of fumagillin against human tumors grown on the CAM of chick embryos support the contention that this method of testing can detect antitumor specificity of agents. Furthermore, the results of repeated tests of fumagillin against two human tumor types clearly indicate the high degree of reproducibility of results obtainable when employing this method for experimental chemotherapy.

Acknowledgments

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Discussion of the Paper

QUESTION: Can you give us the figures for the embryos for blank control? What would the weight of the embryo be at the time of sacrifice? What would the weight of the tumor and embryo be if there were no chemotherapeutic agents, a blank control?

HARRIS: I have explored the effects of these tumors growing on the chorioallantois with respect to their effects on the embryo, and I find that there is essentially no difference between the weights of the embryos that bear tumors and those that do not. There are a few exceptions; that is, if you happen to get a tumor weighing 8 or 9 gm.—and this is quite an exception in the egg—you will find that the embryo will be somewhat smaller. With respect to the weight of the embryo at the time of sacrifice, the average would be about 20 gm. plus or minus 2 or 3 gm. In the tables accompanying my presentation, the figures in the column to the left, the controls, are the untreated. They were given only the diluent, not the compound; the treated chicks given the agent are to the right.

QUESTION: What is the condition of the inhibited tumor? The tumor here that might have grown to be ten or more times as large must have been inhibited. Is there any necrosis in it? Is it as viable as ever?

HARRIS: No. You will find that in the case of fumagillin there are tremendous effects on the cells. The cells would not grow if you tried to grow them elsewhere, that is, in a bioassay. In fact, fumagillin is a compound of microbial origin in which we are particularly interested because of the effects on the cytoplasm and nuclei of the tumor cells. You will find that the cells die and that areas of the tumors become quite necrotic.

Charles P. Dagg (*Brown University, Providence, R. I.*): My brief experience in this field was gained a few years ago in Karnofsky's laboratory at the Sloan-Kettering Institute for Cancer Research, New York, N. Y., where the initial explorations on the utility of egg-grown human tumors in a chemical screening program were made. My associates and I there followed the procedure previously employed by Karnofsky for the study of mouse, rat, and chicken cancer. Small tumor fragments were explanted to the chorioallantois on the eighth day of incubation; on the twelfth day the chemicals were injected into the yolk sac and, on the sixteenth or eighteenth day, the eggs were opened. The chicks and tumors were weighed, histological preparations were made of the tumors and, whenever the tumors appeared to be inhibited, they were assayed by transplantation to eggs or to conditioned rats.

As a result of these investigations it was apparent that the chick's chorioallantoic membrane, as a site for tumor growth, offers a number of unique advantages over the alternatives, namely, tissue culture and conditioned hamsters or rats. With these advantages, however, there are several disturbing features that must be carefully analyzed and controlled before this technique can approach the rigor of method developed for the various mouse tumor screens.

The outstanding advantage is the almost complete absence of antagonism on the part of the chick embryo toward heterologous tissues. Although the chick may begin to respond to certain antigenic substances toward the end of the incubation period, this event customarily occurs from one to several days after the tumors have been removed from the egg. Thus it is possible, by using the chick, to avoid the complications resulting from the combined operations of the agent under consideration and the conditioning agents—irradiation and/or steroids.

Another advantage, although of limited usefulness, is the opportunity to grow two tissues of separate origins in the same egg, thereby determining their relative sensitivities to the same chemical agents. Different human tumors may be compared with each other and human tumors may be compared with the various relatively better-known mouse tumors that are transplantable to eggs. Growing and treating both neoplasms on the same membrane allows the variability due to differences between eggs and undetected experimental errors to be controlled more effectively.

A third exploitable characteristic, which applies only to HEp 3 at present, is found in the marked tendency of this tumor to release metastatic cells that become widely distributed in actively proliferating foci throughout the embryo. Many of these embryos survive the incubation period and hatch, but only a few of the latter develop resistance to the heterologous cancer sufficiently soon to prevent the destruction of vital organs. During the incubation period chemical agents might be assayed for carcinostatic activity against the main tumor mass on the chorioallantois and, also, for activity not only against metastatic nodules in different organs but against different nodules in the same organ.

The rapid rate of growth of the embryo, whose body weight during the period under study increases approximately fourfold as compared with tenfold to one hundredfold increases for the tumors, can be used to distinguish between those agents that affect rapidly multiplying cells in general and those that show some degree of specificity toward cancer cells. There should be little or no confusion created by metabolic similarities between neoplastic and embryonic tissues, since most of the chick's tissues at this stage have completed the major embryonic changes. Developmentally, the chick embryo between 12 and 18 days old is roughly equivalent to stages in the rat from the fetal through the neonatal.

Concerning the specificity of chemical agents: I propose to comment on Harris's previous paper dealing with the activity of various steroids against embryos and human tumors. In several instances in which the treated tumors weighed less than the controls, the host embryos were also markedly inhibited. In some of these cases it is possible that the agent had no direct action upon the tumor and that the tumor was retarded indirectly because the host, which must support the growth of the tumor, was severely affected.

As a screen for the discovery and evaluation of potential chemotherapeutic compounds, the egg-tumor system is subject to many of the difficulties encountered in other screening procedures and has a few others peculiar to itself alone. First, only a small number of the human tumors that can be maintained in tissue culture, in hamsters, or in rats can be successfully propagated on the chorioallantois; therefore the spectrum of tumors that can be employed in this test is limited to a fraction of those available for other methods.

Another possible source of misinterpretation is the great range of tumor sizes, which routinely weigh from 50 to 2000 mg. Under these circumstances I think it would be desirable to implant relatively large numbers of eggs and to subject the data to statistical analysis because, in a small population, one tumor at either of the extremes can have an overbalancing influence upon the average weight.

Egg size and content of vitamins, free amino acids, and other such factors undergo considerable seasonal variations parallel to fluctuations in diet and reproductive capacity. As a consequence, the minimal levels for inhibitory activity by certain types of agents would fluctuate in a similar manner, and estimations of the relative effectiveness of different analogues of the same compound would be reliable only when performed in equivalent groups of eggs.

Misleading results might also arise from quantitative differences in drug sensitivity between mammalian tissues and the avian embryo. It is to be expected that in some instances the chick would tolerate much larger doses of a compound than would a mammalian host or the tumor; therefore antitumor activity could be ascribed to an agent that would be prohibitively toxic in clinical trials. The converse of this situation may occur and important tumor inhibitory agents would be missed altogether. This feature of the system would be important only if it were used as a primary screen and would restrict but not negate its usefulness as a secondary or supporting screen.

At this point I should like to ask three questions of Harris. The first concerns the experiments presented in Part V. Why did you inject the hormones into the yolk sac? I believe Karnofsky has shown that some of these hormones have a much greater effect on the development of the chick when placed on the chorioallantoic membrane. I agree it is possible that these hormones may have direct effects upon the tumors, but that must be proved.

Also, it is not clear to me as to how you determine what dose you are going to give your eggs. I notice that it varied between an LD_{10} and almost an LD_{80} in some cases. Do you recommend an LD_{50} or the maximum tolerated dose, and do you consider the effect upon body weight?

The last question is: Would you compare your data at this point with those obtained by Teller? I think it is interesting to all of us to know how the chick embryo is standing up with the rats in regard to the same chemical agents.

HARRIS: Regarding the method of injecting hormones into the yolk sac instead of into the chorioallantois: if you noticed, we were discussing chorioallantoic implantations. I suppose some people will object to anything you do; there have been considerable objections, for example, to placing tumor tissue into the test agent and then implanting as a chemotherapy method, directly injecting the tumors, and so on. If we were to place the material onto the chorioallantois, which would be precisely where we have the tumors, we should be bathing the tumors in the test agent. By injecting the material into the yolk sac, the agent or the metabolites thereof would have to reach the tumor through the blood or vascular system in the same way that such materials gain access to areas of the embryo.

As for the dose levels, I attempted to emphasize the point that the results we were presenting in the hormone area were quite preliminary. However, we felt that they gave indications as to what one might expect when testing hormones and related agents as antitumor agents. Sometimes we have information available concerning a given agent that has been tested in some other test system, perhaps rat or mouse, and we have found in quite a number of instances that we can make certain calculations and arrive at a reasonably accurate theoretical dose. Once we get into the tolerance range we determine empirically the highest dose the embryos will tolerate. This is not based on weights alone, although weights are one consideration. We try to administer compounds at doses that do not seriously affect the embryos at a therapy level. We look inside the chick to find what effects are evident. Sometimes external features exhibit reduction in feather growth, perhaps edema. The liver reflects evidence of toxicity with some degree of regularity; spotting and necrotic areas may show up there. All of these factors are taken collectively as indicating degrees of effects on the embryo. As I mentioned before, allowing some of these to hatch to see whether they are essentially as well off as are the controls gives additional information.

Regarding the comparison of chick data, we find that, with few exceptions, from the results that Teller has had with what I understand his criteria of a good compound to be, we have not been embarrassed too frequently. I think our results with the chick embryo, using fumagillin, which we consider an interesting antitumor agent, agree with Teller's results with that agent, and I think Cullen presented results with fumagillin that reflect further agreement. There are instances in which we find agents interesting but, in the test with the rat, they may not find this to be the case. The reverse is also true. In the tables we list four different types of tumors: the epidermoid carcinoma, the sarcoma, the adenocarcinoma, and the bronchogenic carcinoma. There are instances in which variations in response existed among them, both qualitatively and quantitatively. We are not by any means disheartened, however. In fact, we are encouraged by such results simply because they support our theme (although it may prove to be erroneous) that cancer is not one disease but many, and that specific agents may have to be directed against specific tumor targets, which may turn out to be certain tumor types. With the aid of biochemists at the Institute, we are trying currently to find out just why some of these differences should exist. We hope these ventures may shed some light on the problem.

QUESTION: What is the average weight of either embryos or chicks that have resulted from drug-inoculated eggs that have not had any tumors transplanted to them?

HARRIS: We have not averaged the drug effects per se because the effects vary. Some agents are not at all toxic, at least not at the doses we have tested, and some are quite toxic. You must consider the degree of toxicity at the levels used. For our therapy levels we use doses that do not appreciably affect the embryo, and thus the average weights of the embryos would approximate those of the controls which, at the time of evaluation, would be about 20 gm.

HELENE TOOLAN (*Sloan-Kettering Institute for Cancer Research, New York, N. Y.*): I should like to ask Dagg, since he has had more experience than many of us with animal tumors on eggs, if he would give us some idea of how the size compares with animal tumors in general. Also, specifically, for example, I believe HS 1 is more uniform than HEp 3, which requires very special conditions of growth, does not grow in tissue culture, and metastasizes widely in the chicken and also in the human host. I think some of its variability may be due to its implantation effect on the egg—the site on the egg if it gets to a blood vessel rather rapidly. In other words, could you compare the growth of for example, HS 1 with some of the well-known mouse tumors, and explain how in general the human tumors compare with the animal tumors? Also, do any of the animal tumors metastasize?

DAGG: The amount of variation with the HS 1 and the HEp 3 is as great as that with the standard animal tumors. This opinion is based on my experience with Sarcoma 180, the Ridgeway osteogenic sarcoma, the EO 771, and the Jensen rat sarcoma. The site of implantation, of course, is extremely important. The tumor is placed on blood vessels in the hope that until it becomes attached to the membrane it will not be washed away by the fluid leaking out of the membrane or included with the tumor. Of course, those that become established early do appear to grow much faster.

I should like to comment upon something Harris said about the direct bathing of the tumor when it is on the chorioallantoic membrane. I do not think that would happen if he were to wait until four days after the tumor had been implanted, because by this time the tumor—a good tumor—would be completely embedded within the chorioallantoic membrane. The only way in which the drug would come into contact with it would be by diffusing through the membrane.

I am not at all clear as to what Harris means by a "positive" test. Is a positive test a tumor inhibition of a certain extent irrespective of what happens to the chick in terms of such factors as weight and hatchability? Or is a positive test defined in terms of what happens to the chick also?

HARRIS: Regarding HEp 3, if I may first refer to what happens to the tumor at four days, I should say that in the majority of instances it becomes embedded in the chorioallantois. When we use the bronchogenic carcinoma or the adenocarcinoma, sometimes a certain amount of critical observation is required to determine whether the tumor fragment, which is on top of the chorioallantois, is still viable and living. Of course, after a period of time, we learn to detect and predict this with a high degree of accuracy.

When we consider a drug as active or positive, we use the arbitrary limits I have shown in my diagram. Inhibition of 25 per cent or less is considered negative. In terms of degree of tumor inhibition, this is possibly influenced by other chemotherapy testing systems. Above 25 per cent inhibition, we consider an agent active. We should keep in mind, however, that "activity" is not as absolute as indicating the degree of activity. There are varying degrees of activity. Compounds we would consider highly active or even very interesting, if we were to use such an ambiguous term, would be compounds that would have given considerable inhibition of the tumors without appreciable effects on the embryo. We take what happens to the embryo into consideration. We think, however, that this system enables one to get some indication, not incontrovertible proof, as to what is happening between the normal and the neoplastic. The tumor represents fast-growing tissue of a neoplastic nature, and the embryo represents fast-growing normal tissue. Differences between the two would be an indication of specificity, but we do not say that this is the most critical type.

CHEMOTHERAPY STUDIES ON TRANSPLANTABLE HUMAN AND ANIMAL TUMORS IN SYRIAN HAMSTERS*

By Alfred H. Handler

Laboratories of Tumor Transplantation, The Children's Cancer Research Foundation, and the Department of Pathology, Harvard Medical School, Children's Medical Center, Boston, Mass.

Experiments with human tumors grown in hamsters have been carried out in some detail in our laboratories in an attempt to formulate a methodology suitable for conducting chemotherapy studies. It is hoped that the use of heterologously grown human tumors in animals will provide a screening method that may be a closer approximation of human neoplasia for screening purposes than is provided by the use of experimental animal tumors in a strictly homologous transplantation system. Although our present studies are based primarily on growth of human tumors in the hamster cheek pouch, they also incorporate homologously grown hamster tumors and heterologously grown animal tumors, in an effort to determine the influence of the host-transplant relationship on experimental chemotherapy.

Eight years ago, in collaboration with Brenton R. Lutz and Donald I. Patt at the Department of Biology, Boston University, Boston, Mass., I transplanted fragments of human tumor tissue to the cheek pouch of the Syrian hamster for the first time.[1, 2] Prior to this, Fulton *et al.*[3] had used the hamster cheek pouch for microcirculatory studies. The cheek pouch was originally selected as a site for tumor transplantation because of its simple structure and the ease with which close observation and volumetric measurements of tumors implanted there can be made.[4]

Since that time we have repeatedly demonstrated the general excellence of these methods for the development of serially transplantable human tumors. Consequently, we now have a variety of tumors derived from children and adults[5] in a state of readiness for experimental use.

Although the cheek pouch of the hamster is highly suitable for the isolation and development of new tumors, its physical dimensions limit use of the preparation for the maintenance of large quantities of stock tumor. This problem has been met by transplanting cheek pouch tumors to intramuscular, intraperitoneal, and subcutaneous sites in the hamster. Intramuscular implants produce extraordinarily large tumors ideally suited for studies requiring tumor tissue in quantity (FIGURES 1 and 2), and they have the added advantage of requiring transplantation less frequently than do cheek pouch tumors.

The present method of transplanting tissues to hamster cheek pouches is a modification of a technique described elsewhere.[4] Hamsters 5 to 8 weeks of age and of both sexes are used routinely for these studies. The hamster is anesthetized with Nembutal† administered intraperitoneally at a dosage of 0.15

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† Veterinary Nembutal Sodium, 60 mg. per cc., was obtained from the Abbott Laboratories, North Chicago, Ill.

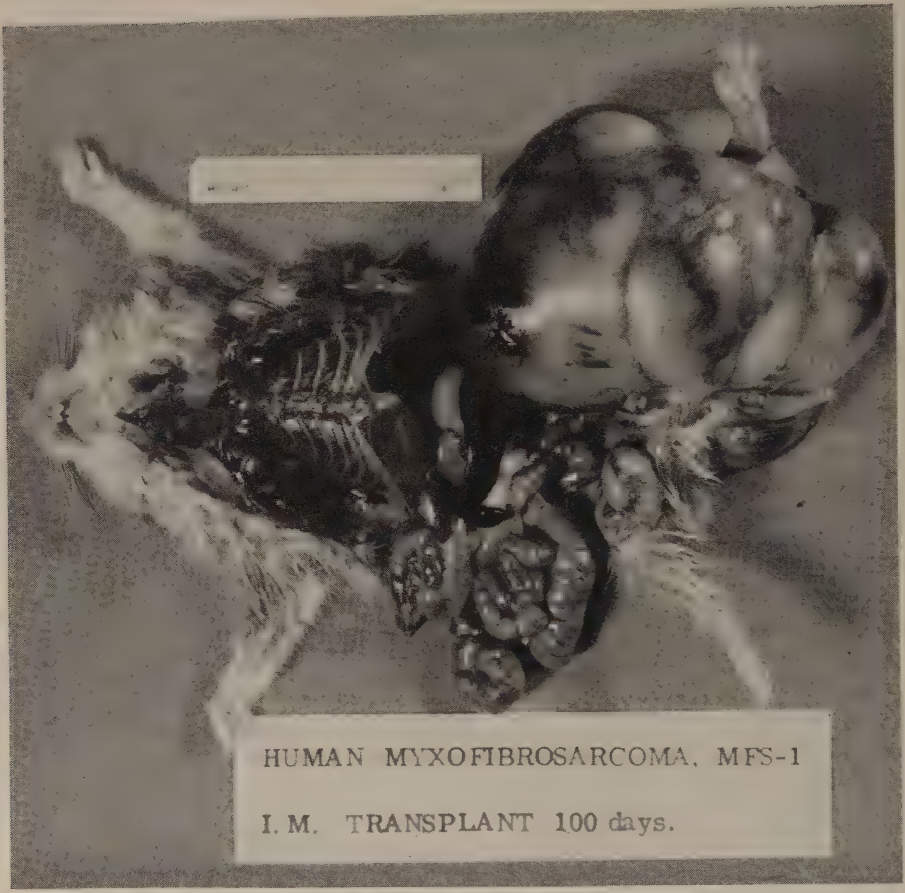

FIGURE 1. Human myxofibrosarcoma (MFS 1) derived from a child, growing in hamster leg muscle. 100 days.

cc./100 gm. of body weight. The cheek pouch membrane is everted with blunt forceps, pinned to a lucite holder, and washed with Zephiran 1:1000. Transplantation is effected in a Blickman hood, where aseptic conditions are maintained as strictly as is practicable. A few small fragments of tumor tissue are implanted by trocar (FIGURE 3). Cortisone acetate is administered in subcutaneous doses of 2 to 3 mg. twice each week, starting on the day the tumor is implanted. Using these methods, we have been able to establish and transfer serially between 60 and 70 per cent of the human tumors obtained for study.

TABLE 1 indicates some of the types of transplantable tumors isolated from adults by means of this technique. It is evident from their latent periods that, when tumors from adults grow in hamsters, they do so within a few days after transplantation. In contrast, as shown on TABLE 2, the latent period for tumors transplanted from children is considerably longer. Although latent

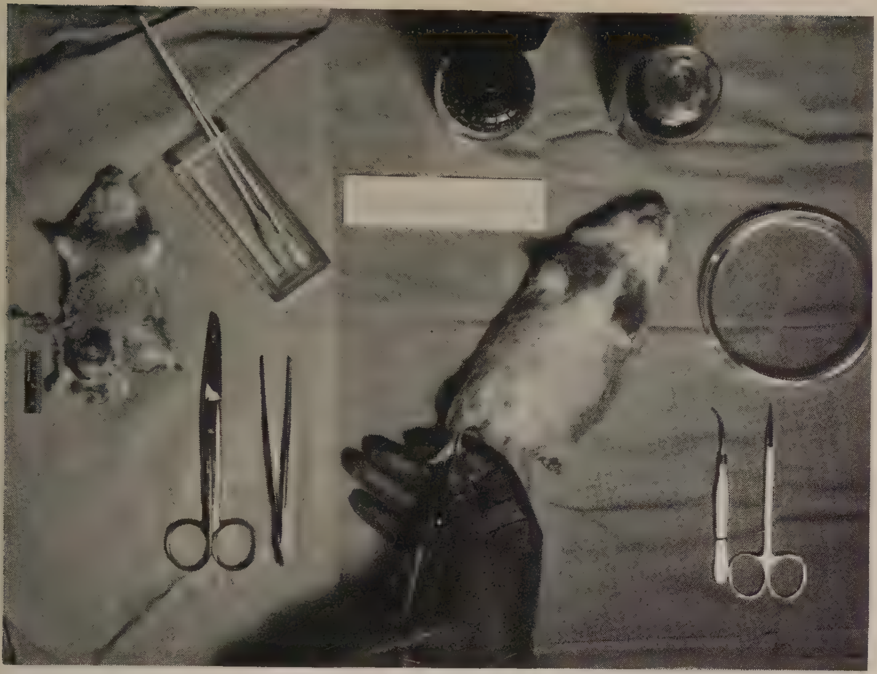

FIGURE 2. Transplantation of tumor into hamster leg. Finely minced tumor suspended in isotonic sterile saline is injected bilaterally. Standard inoculum is 0.5 cc.

periods for tumors from children varied from 1 month to as long as 1 year—in 2 instances of Wilms's tumor—one epidermoid carcinoma from a 17-year-old boy grew after a latent period of only 19 days. This would have been a relatively long latent period for a transplantable tumor from an adult, but it was short for a tumor from a child. Further transplant studies using tumors from different age groups should be made.

We have determined the following design as the most suitable for our present experimental chemotherapy investigations. Tumor fragments are first im-

TABLE 1

HUMAN TUMORS ISOLATED FROM ADULTS AND PERPETUATED BY PASSAGE IN CHEEK POUCHES OF CORTISONE-CONDITIONED GOLDEN HAMSTERS*

Number	Tumor	Latent period (days)
3	Fibrosarcoma	5–14
10	Melanoma	5–14
5	Epidermoid carcinoma	6–14
1	Carcinoma of breast	9
1	Teratoma	10
1	Carcinoma of pancreas	7

* The latent period after initial transplantation into cheek pouches is indicated.

TABLE 2

HUMAN TUMORS ISOLATED FROM CHILDREN AND PERPETUATED BY PASSAGE IN CHEEK POUCHES OF CORTISONE-CONDITIONED GOLDEN HAMSTERS*

Number	Tumor	Latent period (days)
6	Osteogenic sarcoma	30–100
1	Ewing's sarcoma	60
2	Rhabdomyosarcoma	60–75
2	Myxofibrosarcoma	60–100
1	Angiosarcoma	30
1	Carcinoma of parotid	30
1	Epidermoid carcinoma (17-year-old ♂)	19
1	Neuroblastoma	45
14	Wilms's (embryoma of kidney)	30–360
4	Teratoma	30–90

* The latent period after initial transplantation into cheek pouches is indicated.

planted into the right cheek pouches of either agouti or albino hamsters. The implants are permitted to grow and become vascularized. Cortisone acetate is administered to the hamsters, according to the growth requirements of the individual tumors. After the implants have become well established, hamsters are paired according to the size and vascularity of their tumors. One hamster of each pair receives the test compound; the other serves as a control and receives only the compound vehicle. Daily subcutaneous or intraperitoneal injections of test compounds are continued as long as the growth pattern of the tumor permits, that is, until necrosis of the control tumor is first observable. Experiments are terminated the day following the last injection of compound. Tumors are then photographed, measured, and fixed for histological sectioning. Autopsies are performed. Evaluation of the effectiveness of a compound is based upon the gross size and appearance and the microscopic appearance of the tumors treated, as compared with the gross size and appearance and the microscopic appearance of control tumors. The preparation and interpretation of histological material are under the direct supervision of Sidney Farber, who has also given us the benefit of his experience in the planning of these experiments.

We have tested the effectiveness of compounds belonging to the phosphoramide, dihydrotriazine, actinomycin,[6] and folic acid antagonist groups against a myxofibrosarcoma from a child, and 2 malignant melanomas, 2 epidermoid carcinomas, and a sarcoma from adults. Compounds have been tested also against mouse and hamster tumors transplantable in hamsters.

Actinomycin D was selected for detailed study, since Farber *et al.*[7] had already reported the carcinolytic activity of this substance in mouse tumor systems and, in addition, had described temporary effects against certain tumors in man, such as rhabdomyosarcoma, Wilms's tumor, and various forms of lymphoma.

TABLE 3 summarizes the effect of actinomycin D on the tumors utilized in these experiments. These tumors were selected because they represent human tumors from children and adults, as well as animal tumors, which grow in ham-

TABLE 3

EFFECTS OF ACTINOMYCIN D ON HUMAN AND ANIMAL TUMORS TRANSPLANTABLE IN CHEEK POUCHES OF GOLDEN HAMSTERS*

Neoplasm	Conditioning	Dosage†			Remarks
		MTD	1/2 MTD	1/4 MTD	
ME 1: human amelanotic melanoma (adult)	Cortisone	+	+	+	Thio-TEPA active at MTD
MFS 1: human myxofibrosarcoma (child)	Cortisone	+	−	−	Thio-TEPA active at MTD
KB: human carcinoma of jaw (Eagle)‡	Cortisone	+			
S 180: mouse sarcoma‡	Cortisone	+			
P 1534: mouse lymphatic leukemia	None	+	−		Aminopterin inactive at MTD
	Cortisone		−		
PET 1: hamster sarcoma	None	−			TEM inactive at MTD
	Cortisone	−			

* The effects of maximum tolerated dosage of a few control compounds against some of the tumors are indicated.

† Dosages of actinomycin D, maximum tolerated dose in hamsters: MTD = 125 γ/kg./day for 7 or 8 days; ½ MTD = 62.5 γ/kg./day for 7 or 8 days; ¼ MTD = 31.25 γ/kg./day for 7 or 8 days. The + indicates that the dosage was active in controlling growth; the − indicates that the dosage was inactive in controlling growth.

‡ Transplanted as quantitated inocula of cells grown in tissue culture by George E. Foley, The Children's Cancer Research Foundation, Boston, Mass.

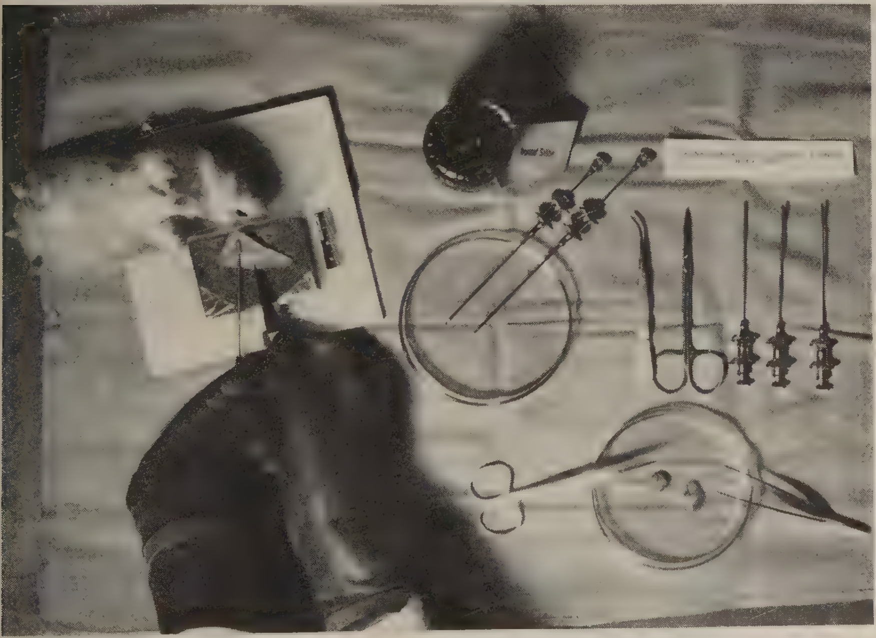

FIGURE 3. Transplantation of human tumors into hamster cheek pouch by means of a trocar.

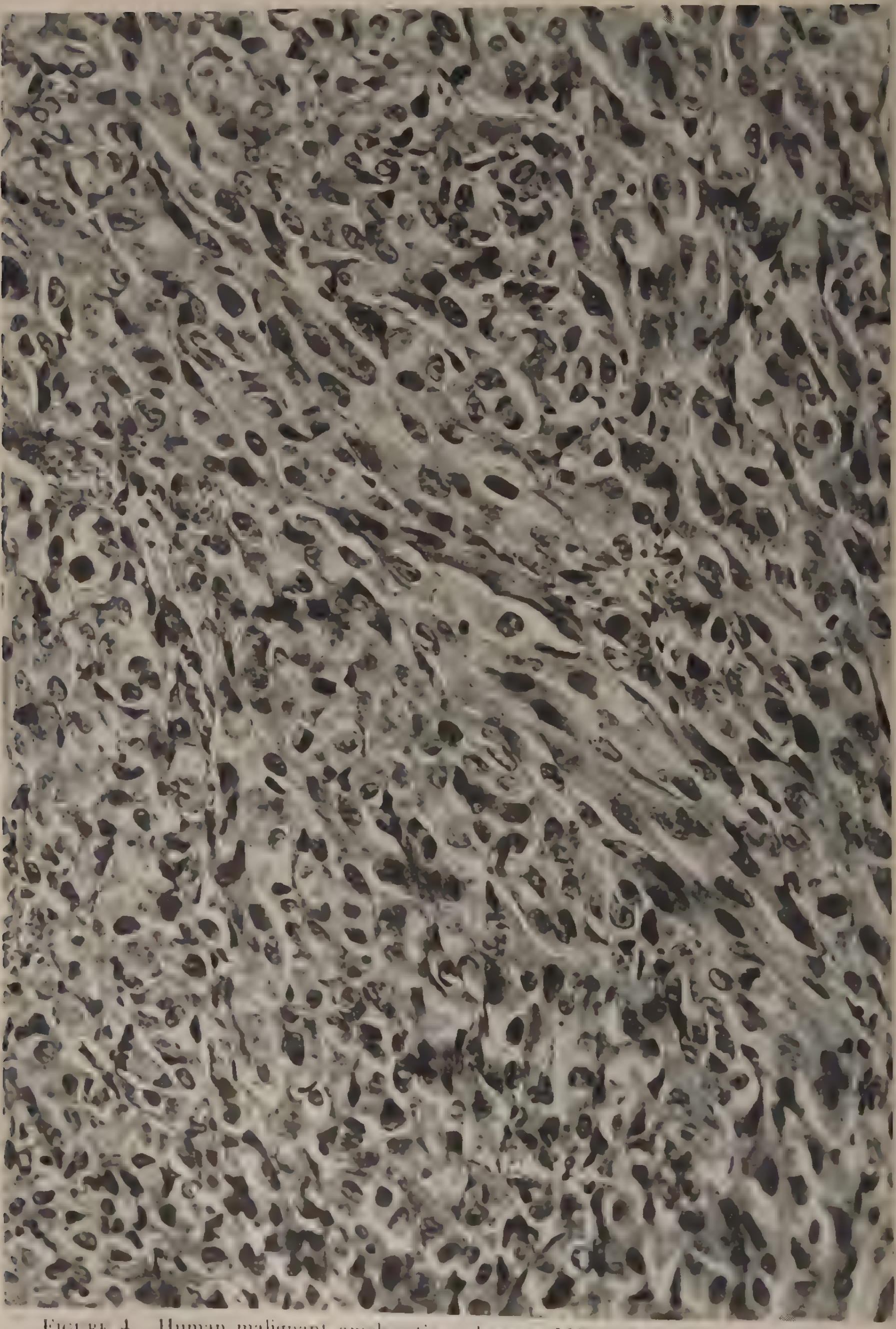

FIGURE 4. Human malignant amelanotic melanoma ME 1 derived from an adult, actively growing in cheek pouch of cortisone-conditioned hamster for 23 days. Saline treated control animal. Hematoxylin and eosin. ×115.

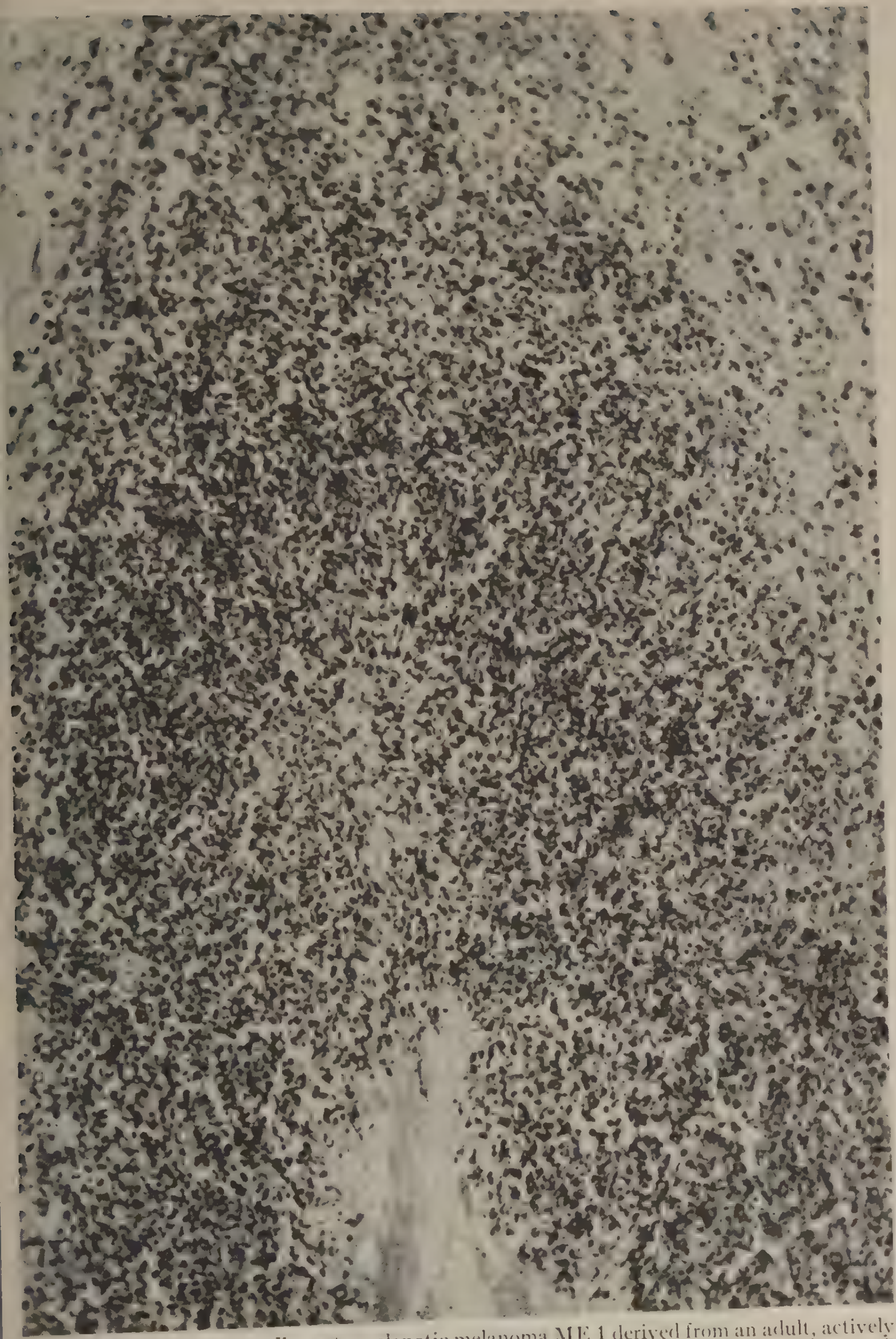

FIGURE 5. Human malignant amelanotic melanoma ME 1 derived from an adult, actively growing in cheek pouch of hamster treated with MTD actinomycin D, 125 γ/kg./day for 7 days. At this dosage, actinomycin D effectively destroyed the melanoma. Hematoxylin and eosin. ×115.

sters with and without the aid of cortisone conditioning. Mouse sarcoma 180 (S 180) and human carcinoma of the jaw (KB), isolated, respectively, by George E. Foley[8] and Harry Eagle,[9] were utilized because they demonstrate the utility for chemotherapy studies of tumor cells isolated in cell culture and transplanted to hamsters as quantitated inocula of trypsin-dissociated cells. TABLE 3 also shows the effects of other compounds administered at the maximum tolerated dose (MTD) against some of these tumors. From previous unpublished studies we have determined that in hamsters the MTD of actinomycin D is 125 γ/kg./day for 7 or 8 days; of Thio-TEPA, 7 mg./kg./day for 8 days; of TEM, 700 γ/kg./day for 7 days, and of Aminopterin, 700 γ/kg./day for 6 days.

When human malignant amelanotic melanoma (ME 1) was transplanted into the hamster cheek pouch and treated with actinomycin D at the MTD, the tumor was completely destroyed (FIGURES 4, 5, and 6); this effect was observed on newly established tumors as well as on tumors that had reached large size prior to therapy (FIGURE 7).

A marked antitumor effect was also observed against ME 1 at one half the MTD of this compound (FIGURE 8). At one fourth the MTD, actinomycin D did not destroy the tumors, although it did arrest growth. Since this experiment was terminated when the control tumors began to break down, we do not know whether the arrested tumors would have continued to grow after the cessation of therapy.

When the myxofibrosarcoma (MFS 1) was treated with the MTD of acti-

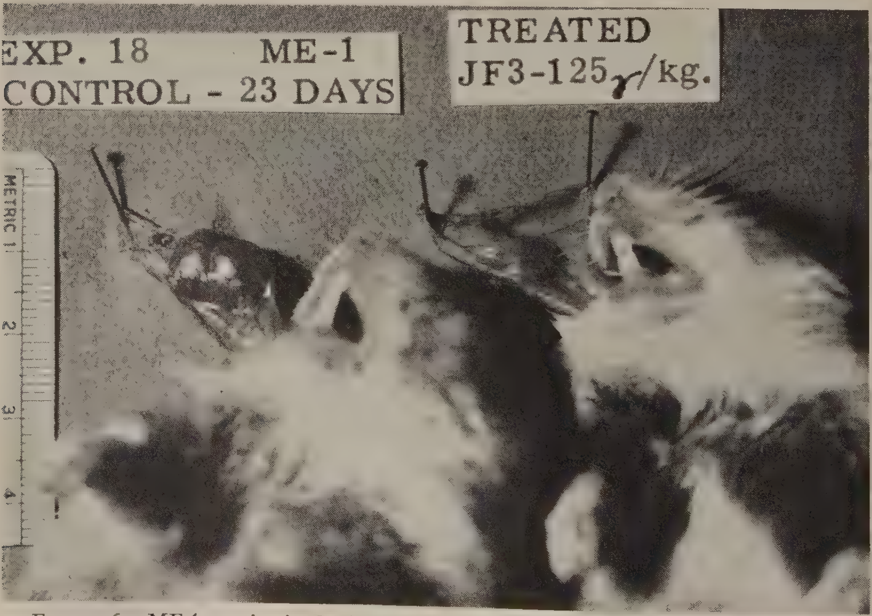

FIGURE 6. ME 1 growing in cheek pouches of cortisone-conditioned hamsters for 23 days. Left, saline-treated control; right, treated with actinomycin D, 125 γ/kg./day for 7 days.

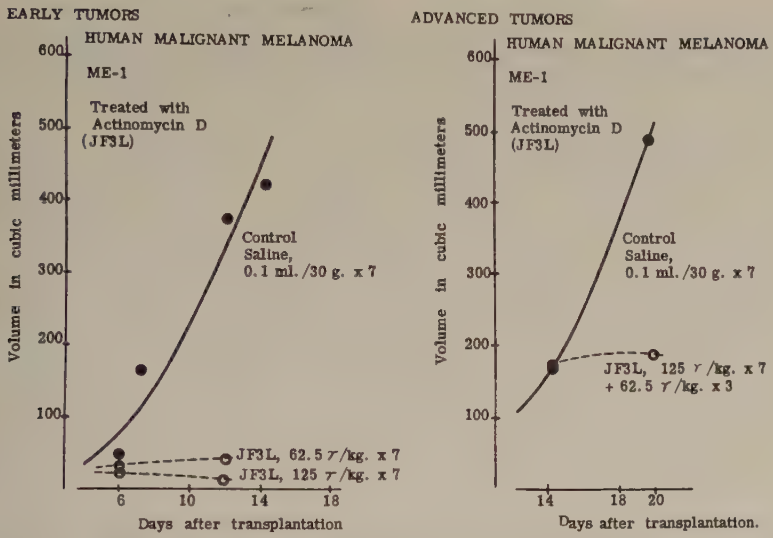

FIGURE 7. Effects of actinomycin D against ME 1 when transplanted into cheek pouches of cortisone-conditioned hamsters. Compound was tested against tumors transplanted less than 1 week prior to therapy and against advanced tumors.

nomycin D, the tumor cells were destroyed (FIGURES 9 and 10). However, at one half the MTD of actinomycin D, in spite of a slight gross antitumor effect, little or no histological effect was observable.

P 1534, a transplantable lymphatic leukemia indigenous to DBA/2 mice,[10] was transplanted to hamsters in the present chemotherapy study as a representative of those tumors heterologously transplantable in hamsters but requiring no cortisone conditioning. The MTD of actinomycin D had a marked antitumor effect against hamster-grown P 1534, as it does when this leukemia is grown in the mouse strain of origin.[7] Two control compounds, cortisone acetate and Aminopterin, were ineffective against P 1534 in the hamster. Tumors treated with these compounds were indistinguishable from saline-treated control tumors both grossly and histologically. The lack of effect of Aminopterin on P 1534 grown in hamsters was of particular interest, since nontoxic dosages failed to show any activity against P 1534 leukemia in DBA/2 mice.*

Discussion

Actinomycin D at the MTD in hamsters was found to be effective against all tumors used in this study, with the exception of hamster sarcoma (PET 1).[11] Varying the dosage of this compound indicated differential dose-tumor responses. In a number of acute experiments, TEM, Thio-TEPA, and Aminopterin were employed as drug controls and indicated differential tumor-compound responses. In experiments utilizing P 1534 and PET 1, which grow in hamsters without cortisone conditioning, the effect of actinomycin D seemed to be independent of the cortisone effect, indicating that, when retardation of

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* M. N. Swaffield and C. L. Maddock, personal communication in 1958.

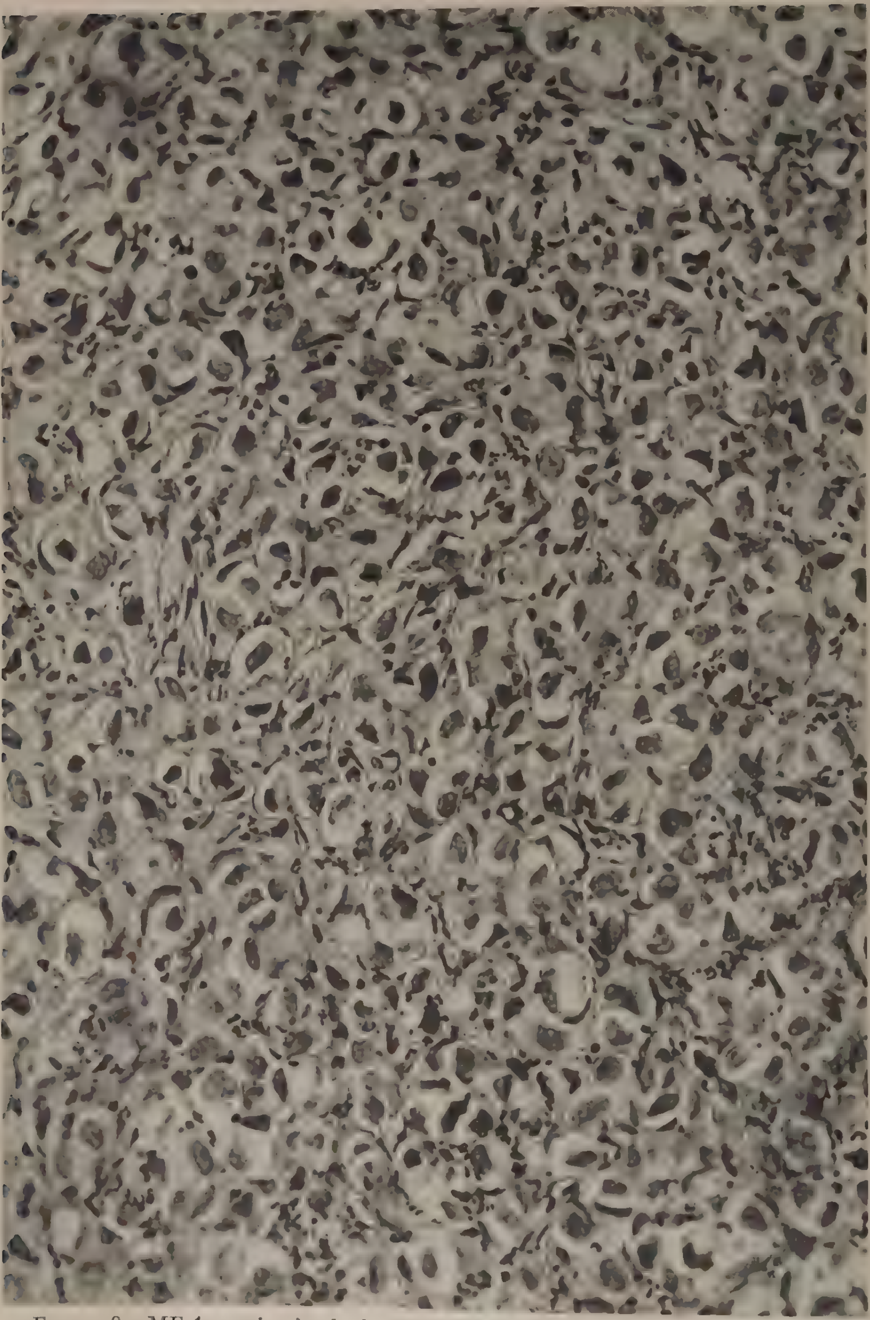

Figure 8. ME 1 growing in cheek pouch of hamster for 23 days and treated with ½ MTD actinomycin D, 62.5 γ/kg./day for 7 days The compound at this dosage has a marked antitumor effect. Hematoxylin and eosin. ×115.

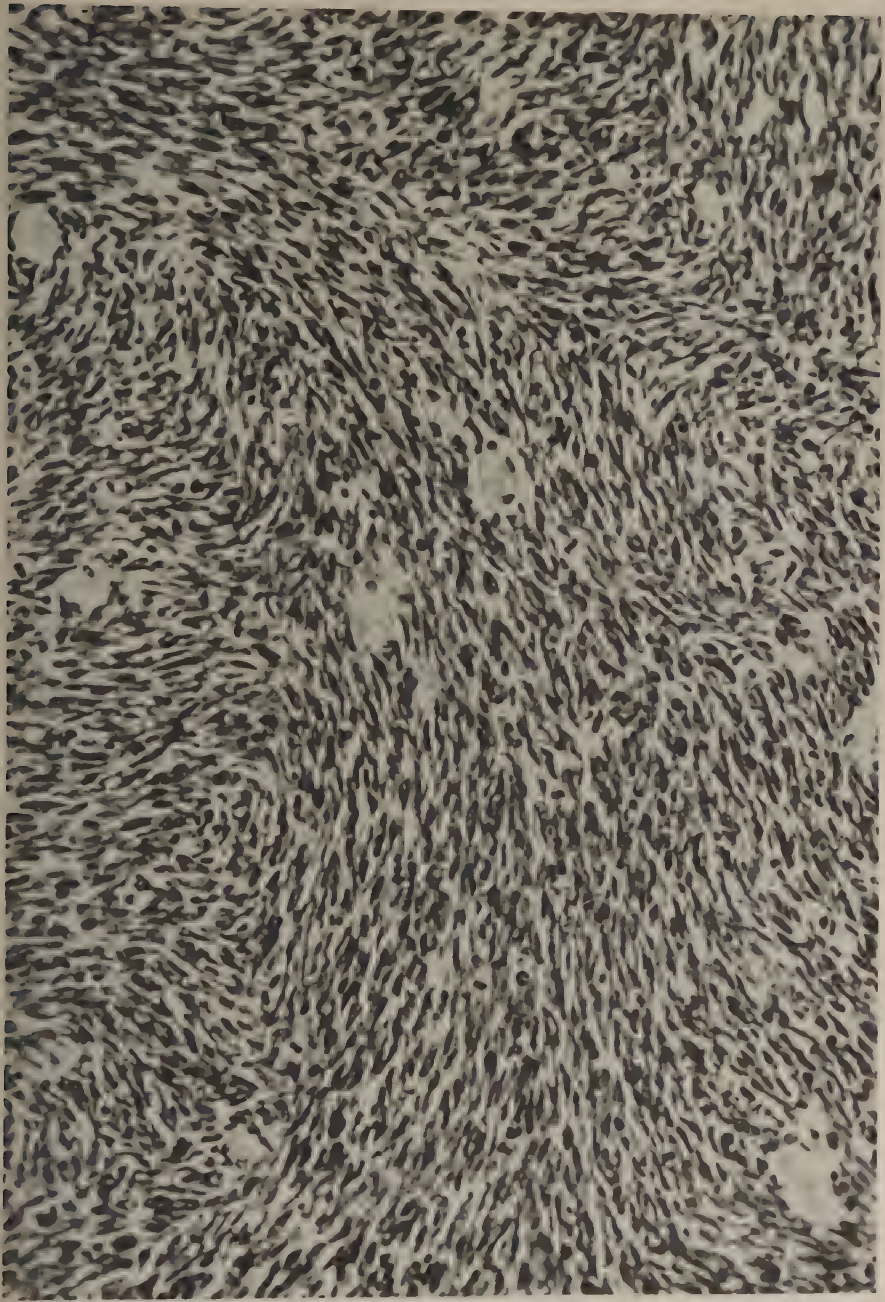

FIGURE 9. MFS 1 actively growing in cheek pouch of cortisone-conditioned hamster for 17 days. Saline-treated control. Hematoxylin and eosin. ×115.

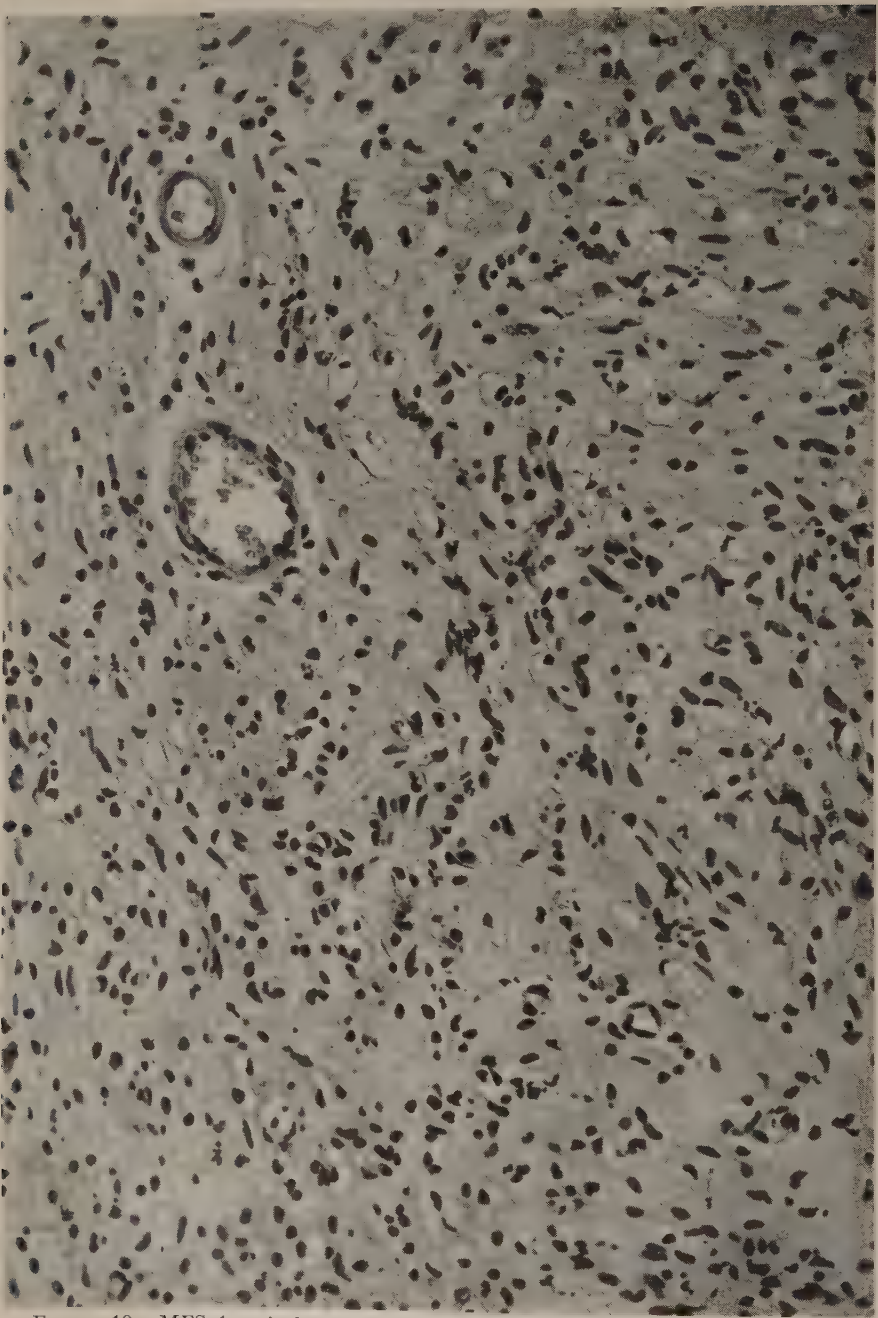

Figure 10. MFS 1 actively growing in cheek pouch of hamster treated with MTD actinomycin D, 125 γ/kg./day for 7 days. The tumor was destroyed at this dosage. Hematoxylin and eosin. ×115.

growth occurred, it was not due to interference with the host-transplant relationship.

Additional experiments are being conducted in our laboratories to develop new techniques, such as growth of human tumors in mice bearing homologously transplanted ACTH-secreting pituitary tumors.[12] By means of this technique we have been able to grow human tumors directly from the patient, as well as tumors that have been isolated in hamsters. Tissue-culture cell lines also have been successfully implanted in these mice, which require no exogenous cortisone conditioning. In time, this mouse pituitary tumor-human tumor system in LAF_1 mice may be useful for studying potential anticancer agents.

Experiments are also being conducted to establish a greater variety of human tumors in hamsters; these will be added to the large number and variety of transplantable neoplasms already established in our laboratories.

Summary

A variety of human tumors from both children and adults has been isolated in the cheek pouches of cortisone-conditioned Syrian hamsters. Growth and perpetuation of these tumors in hamsters have provided a method with which to study the biology of cancer. In our laboratories, experiments have demonstrated the suitability of the hamster-human tumor system for detailed study of known and potential anticancer agents. Further experiments have indicated that these methods may be used also for routine chemotherapeutic screening.

Compounds belonging to the phosphoramide, dihydrotriazine, actinomycin, and folic acid antagonist groups were tested in hamsters for their effectiveness against such human tumors as a myxofibrosarcoma isolated from a child, as well as two malignant melanomas, two epidermoid carcinomas, and a sarcoma isolated from adults. Compounds also were tested against mouse and hamster tumors transplanted into hamsters, and against tumor cells isolated in tissue culture and transplanted into hamsters. Evaluation of the effectiveness of a compound was based upon the gross size, appearance, and histology of the tumors, as compared with the size, appearance, and histology of the control tumors.

At various dosages, actinomycin D, markedly retarded the growth of several of the tumors used in this study. Therefore detailed investigations were made with this compound. Results of these investigations have thus far demonstrated: (1) that a tumor responds differently to different compounds; (2) that a tumor responds differently to different doses of a given compound; and (3) that different tumors differ in their responses to a given compound.

Acknowledgments

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Discussion of the Paper

SHELDON C. SOMMERS (*Massachusetts Memorial Hospitals, Boston, Mass.*): Alfred H. Handler was a member of the original group at Boston University where the hamster cheek pouch was being used for biological investigations. He exploited this method for human tumor transplantations and, as his paper indicates, he has continued since that time to utilize this technique successfully in transplanting a variety of human cancers and embryonic tissues. For chemotherapeutic experiments in our laboratory at the Massachusetts Memorial Hospitals, this technique was chosen in preference to the others available for cancer chemotherapeutic testing, because it appeared possible for any intelligent individual to learn within six months to transplant tumors in this way effectively, and because the tumors could easily be examined at intervals with respect to their size, shape, color, consistency, and vascularity. This method is useful in studying tumor biology and tumor-host relationships, as well as in chemotherapy.

As a pathologist in almost daily contact with the problems of cancer in human patients, I can state with assurance that in the transplant the human tumor maintains its individuality both architecturally and cytologically. Since these tumors grow differently in different individuals, the experienced transplanter can tell one from another, although they may be microscopically of the same cell type.

Besides the tumors already discussed, there is now available in the laboratories in the Boston area a rather wide variety of tumor types, including epidermoid carcinomas of the skin and salivary gland, adenocarcinomas of the endometrium, breast, and ovary, undifferentiated carcinomas of the prostate and thyroid, malignant melanomas, gliomas, and Wilms's tumors. Consequently, anything that might be used effectively on a certain cell type could be applied to this wide variety of tumors.

These tumors with their individual peculiarities appeal to the pathologist for chemotherapeutic testing somewhat more than do the undifferentiated types of animal tumors, some of which have been grown for 40 years or more, during which period they have been subjected to natural selective processes. Whatever may be the reasons, these older tumors have lost much of their microscopic individuality and, by inference, perhaps some of their other specific characteristics. If one could treat these more undifferentiated animal cancers successfully, perhaps one could cure any cancer, but it may not be practicable to consider any cancer potentially curable. There are shortcomings in using the hamster, which requires conditioning with cortisone; moreover, the tumors regress on the average of three weeks after transplantation. However, these disadvantages should not be taken too seriously as they are compensated for by the advantages of the method. There is evidence that the human cancer host undergoes a relatively long period of abnormality or conditioning before the tumor develops, and the conditioned human host and the hamster host are perhaps both abnormal in this respect.

Finally, the problem of exploiting these human tumors for chemotherapeutic testing might well have a broader base. It would be advantageous to be able to test a variety of tumors of the same cell type against one or more selected drugs. If only a minority of malignant melanomas responded to a drug, one could test this drug against a number of melanomas and, possibly, one out of ten might respond. It is hoped that the talented individual who learns to transplant tumors from the patient to the animal and then carry them through those first few critical transplant generations will continue to discover new tumors. Now that methods have been described for freezing tumors to preserve them, the individual laboratory need not be overwhelmed with housekeeping duties involved in maintaining tumors. A constant supply of new tumors will, it is hoped, interest others in the field of human tumor transplantation as a method of chemotherapeutic testing.

QUESTION: How well do normal tissues or embryonic tissues, normal adult tissues, or embryonic tissues go into the average hamster cheek pouch?

ALFRED H. HANDLER: We have transplanted both human and mouse embryonic tissues to the hamster cheek pouch and have found that many of them do very well. For example, tissue from the brain, liver, skin, cartilage, and placenta, all did very well. We transplanted heterologous normal adult tissue and it has consistently failed to grow.

QUESTION: What was the size of the embryos?

HANDLER: The embryos we transplanted were about 40 mm.

QUESTION: How serious was the incidence of regression mentioned by Sommers?

SOMMERS: I believe that only in Toolan's laboratory with the HEp 3 did the tumors continue and kill the hamsters when they were implanted in the cheek pouch. In my own laboratory, taking some interesting observations in Handler's work and in Patterson's experiments at Boston City Hospital, no hamsters were killed by the overgrowth of tumor. Therefore, in my own experience, this is an inevitable end result of the cheek pouch transplantation.

Question: Would Handler review briefly the transplanting of tumors in mice?

Handler: We transplanted some of the tumors that we had established in hamsters to LAF1 mice bearing transplants of ACTH-secreting tumors. These were the tumors that Furth discussed, transplantable ACTH-secreting pituitary tumors, and we found that if we transplanted one of these tumors from the hamster to these mice the tumor would grow without giving the mice cortisone. In other words, they received no exogenous cortisone. Perhaps they were producing their own cortisone. Later we went to human tumors directly from humans and we found that they grow just as well. We have not done any further work with this, but we certainly hope to do so soon.

THE HUMAN TUMOR IN THE MOUSE*

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Introduction

The successful transplantation of heterologous tumors was achieved by Murphy[1, 2] more than 40 years ago. Beginning in 1938, Greene[3-6] clearly demonstrated that human tumors can be heterotransplanted successfully to the anterior chamber of the eye of the guinea pig, rabbit, and mouse, although with great variation in success. In general, the growth of the tumors was poor. Hurst *et al.*[7] in 1939 cultivated a variety of human neoplasms on the chorioallantois of chick and duck embryos, but the growth was disappointingly small and insufficient to allow serial transfer. Then, in 1951, an important advance in the technique of human tumor transplantation was achieved by Toolan.[8] On the basis of Murphy's results[1] she irradiated rats and mice before implantation and obtained positive evidence of tumor growth. Even more important, she succeeded in transferring 2 to 3 per cent of these tumors for many generations in X-irradiated and/or cortisone-treated rats and hamsters.[9]

Many reports have now appeared concerning the transfer of human tumors, primarily in rats and hamsters.[10-14] The mouse, however, appears to have been almost neglected as a possible host.

In our experiments, attempts were made to transfer serially the human epidermoid carcinoma HEp 3 into treated or conditioned mice. These studies were conducted in the hope of establishing mouse-grown tumors that could be used for the evaluation of anticancer drugs, chemicals, and regimes. Experiments in this direction are now in progress.

Since many data showed the importance of the site of implantation to the growth of both the homotransplant[15-18] and the heterotransplant,[19, 20] the tumor was transplanted to three sites. In addition, an attempt was made to block the immunological response of the host by methods other than the usual combination of X ray and cortisone.

In a third series of experiments, which was preliminary in nature, we compared the transplantability of HEp 3 to mice of different inbred strains possessing different histocompatibility (H) genes in the H-2 locus.[21, 22]

Materials and Methods

The mice used in our experiments were females weighing about 15 gm., a noninbred Swiss strain‡ and the following inbred strains:§ C57BL/10, DBA/2, BALB/c, and C3H/JAX.

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† Visiting Research Fellow.

‡ This strain was obtained from Taconic Farms, Germantown, N. Y.

§ These strains were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.

The tumor was the human epidermoid carcinoma HEp 3, which was taken in 1953 for heterotransplantation by Toolan[9] from a metastatic epidermoid carcinoma, grade III, primary in the buccal mucosa of a human patient. For our experiments the tumor was usually taken from rats treated with X irradiation and cortisone.

Total-body X irradiation consisting of 1 dose of 50 r, 100 r, or 150 r was given, usually between 1 and 3 days—seldom between 4 and 5 days—prior to implantation of the tumor. Two X-ray machines were used. The first was a Westinghouse 250 kvp. X-ray rotation therapy machine with 1 tube at 180 kvp.—15 mAmp. with added filter of 0.28 mm. Cu to give a one half-value layer of 0.80 mm. Cu. The dose rate was 90 r/min. in air. The second machine was was a 2-tube 180 kvp. at 25 mAmp. with each tube filtered (0.19 mm. Cu, 0.147 mm. Cu) to give a one half-value layer of 0.80 mm. Cu. The dose rate was 86 r/min.

The substances tested for their modification of the immunological response of the host were:

(1) Cortisone* diluted with distilled water in a concentration of 1 mg./0.1 cc., usually a dosage of 1.5 mg., injected subcutaneously immediately after transplantation, and 2 or 3—seldom 4—subsequent injections of 1.0 mg. each on alternate days following the date of implantation.

(2) Corticosterone suspended in saline in a concentration of 2 mg./0.1 cc. injected subcutaneously, a dosage of 4 mg. immediately after transplantation, and 3 additional injections of 3 or 4 mg. each on alternate days following the date of implantation.

(3) Trypan blue suspended in saline in a concentration of 1 mg./0.1 cc. injected subcutaneously, in dosages ranging between 3 and 5 mg.

(4) Thorotrast, 25 per cent thorium dioxide in saline, injected intraperitoneally in a dosage of 0.15 cc. (37.5 mg.) after transplantation, and 2 or 3 injections of 0.1 cc. (25 mg.) on alternate days following transplantation.

The technique of transplantation followed closely that of Toolan,[9] and the amount of the transplant was 0.5 cc. of a 50 to 70 per cent suspension of tumor tissue.

The growth of the tumor was considered positive if the amount of healthy tumor material after 7 or more days was greater than that inoculated, and if the histology indicated that there were viable proliferating tumor cells.

The tumors often regressed and disappeared after 3 or 4 weeks, although sometimes the animals died with large tumors.

Results

The heterotransplantability of HEp 3 in different sites of inoculation. In this series of experiments, the transplantability of the human tumor HEp 3 was compared in 3 different sites of inoculation: subcutaneous (S.C.), intramuscular (I.M.), and intraperitoneal (I.P.) in identically treated mice. The subcutaneous

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* Cortisone in the form of Cortone Acetate was supplied by Merck Sharp & Dohme, Philadelphia, Pa.

TABLE 1
THE TRANSPLANTABILITY OF HEp 3 IN DIFFERENT SITES IN SWISS MICE

Treatment		Site*	Generations of growth	No. mice used
X-ray, r	Cortisone, mg. (avg.)			
50	5.0	S.C.	1	25
50	5.0	I.P.	2	25
50	5.0	I.M.	2	25
100	4.5	S.C.	2	49
100†	4.5	I.P.	4	52
100‡	4.0	I.M.	8	84

* The sites of inoculation were subcutaneous (S.C.), intraperitoneal (I.P.), and intramuscular (I.M.).
† Donor was the Swiss mouse, first generation.
‡ Donor was the DBA/2 mouse, first and fourth generations.

inoculation was made in the right flank and the intramuscular inoculation was made in the left leg.

The results, summarized in TABLE 1, show that when the animals received 100 r and 3.5 to 4.5 mg. of cortisone, the total number of generations in which the tumor grew was only 2 when the tumor was transferred S.C., 4 when it was transferred I.P., and 8 when it was transferred I.M.

The best results with this treatment of the host were achieved in the I.M. inoculation, as shown in TABLE 1.

A comparison of the heterotransplantability of HEp 3 in different strains of mice. The HEp 3 tumor was transferred to treated mice of the 4 inbred strains, C57BL/10, C3H/JAX, BALB/c, and DBA/2, and the Swiss noninbred strain.

The results of this experiment are presented in TABLE 2. The data show that with the same treatment, 100 r and 3.5 to 4.5 mg. cortisone, the best results,

TABLE 2
THE TRANSPLANTABILITY OF HEp 3 IN DIFFERENT STRAINS OF MICE

Strain of mouse	H-2 allele	Treatment		Site	Generations of growth	No. mice used
		X-ray, r	Cortisone, mg. (avg.)			
C57BL/10	H-2^b	50	4.5	S.C.	2	13
C57BL/10	H-2^b	50	4.5	I.M.	2	19
C57BL/10	H-2^b	100	4.5	I.M.	2	10
DBA/2	H-2^d	50	4.5	S.C.	2	16
DBA/2	H-2^d	50*	4.0	I.M.	15	98
BALB/c	H-2^d	100	3.5	I.M.	5	52
C3H/JAX	H-2^k	100	3.5	I.M.	3	17
Swiss	Noninbred	50	5.0	S.C.	2	25
Swiss†	Noninbred	100	4.0	I.M.	8	84

* Irradiated with 100 r from fifth to fifteenth generations.
† Donor was the DBA/2 mouse, first and fourth generations.

TABLE 3

THE TRANSPLANTABILITY OF HEP 3 TO SWISS MICE UNDER DIFFERENT HOST TREATMENTS

Treatment			Site	Generations of growth	No. mice used
	Substance				
X-ray, r	Name	Amt., mg. (avg.)			
None	None	—	S.C.	0	25
None	None	—	I.M.	0	12
None	Trypan blue	4.0	S.C.	0	8
None	Trypan blue	3.0	S.C.	1	11
100	Trypan blue	5.0	S.C.	1	15
150	Trypan blue	5.0	S.C.	1	8
None	Cortisone	4.5	S.C.	1	33
None	Cortisone	4.5	I.M.	2	10
100	Cortisone	4.5	S.C.	2	49
100	Cortisone	4.0	I.M.	8	84
100	Thorotrast	0.4	I.M.	2	13
None	Thorotrast plus cortisone	0.4 3.5	I.M.	4	23
None*	Corticosterone	16.0	S.C.	1	5
100	Corticosterone	15.0	S.C.	3	22
100*	Corticosterone	16.0	I.M.	6	28

* Donor was the Swiss mouse, second generation.

that is, the greatest number of tumor transfer generations, were achieved in the DBA/2 strain –15 generations and still actively growing –which possesses the H-2^d allele; however, the tumor grew only 5 generations in the BALB/c strain, which possesses the same H-2^d allele.

Comparatively good results were achieved in Swiss mice, although the tumors stopped growing for unknown reasons after 8 generations (TABLE 2).

The transplantability of HEp 3 in Swiss mice under different host treatments. In addition to the usual treatment of the hosts with X irradiation and cortisone, an attempt was made to block the immunological responses with three other substances: corticosterone, trypan blue, and Thorotrast.* The substances were administered either alone or with additional treatment of X irradiation or cortisone. The results are presented in TABLE 3. The data indicate that none of these substances gave better results than those achieved with the X irradiation and cortisone treatment, although the results with X ray and corticosterone and even with Thorotrast and cortisone appear to be worthy of further study.

Discussion

Inoculation site. Our results show that the heterologous tumor grew more abundantly and could be transferred for more generations in the intramuscular site, which is believed to have a richer blood supply, than in the sites assumed to have poorer blood supplies.

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* A proprietary colloidal suspension of thorium dioxide.

Two factors may be of importance in the interpretation of these results: the blockage of the immunological response of the host and a rich blood supply, that is, better nutrition to the tumor.

The variability of successful heterologous transplantation to the intramuscular, subcutaneous, and intraperitoneal sites of implantation in treated mice resembles that found in the transplantation of homologous tumors to untreated mice,[16] with the exception of the intraperitoneal site, where different genetic requirements from the tumor may be involved.[23]

Strains of mice. The importance of the role of H genes in determining the fate of tumor transplants in inbred strains of mice is firmly established.[22] It is known, too, that the H genes exist in the mouse at different genetic loci and that the H-2 locus, which is thought to be a "strong" locus, exists as a series of alleles[24] or pseudoalleles.[25]

The purpose of our studies was to discover whether a correlation exists between the degree of transplantability of the heterologous tumor and the H-2 alleles of similarly treated hosts.

Our findings did not confirm such a correlation. The HEp 3 could be transferred to the C57BL/10 strain with the H-2^d allele and to the C3H strain with the H-2^k allele for only 2 and 3 generations, respectively. On the other hand, the same tumor was transferred to the DBA/2 strain with the H-2^d allele for as many as 15 generations, and it is still growing, while in the BALB/c strain, which possesses the same H-2^d allele, it grew for only 5 generations.

We assume, therefore, that hosts of different strains do not display the same blocking of immunological responses even though they have received the same treatment and possess the same H-2 allele.

Furthermore, our unpublished data indicate that other human tumors differ in their ability to be transferred to mice of the same strains treated in the same manner as reported here, that is, a sarcoma, HS 1.

Immunological blocking agents. The immunological response of the host to homologous and heterologous tumor transplants is not yet fully understood. Some believe that this response is effected through the humoral antibodies; others point out the importance of cellular mobilization[26, 27] or a combination of both; still others think that other means exist, for example, an allergiclike response.[28, 29]

In an attempt to block the defensive mechanism of Swiss mice against the human tumor HEp 3, we used the following means and substances (either alone or in combinations) which are known to lower the host resistance to tumor grafting: X ray, cortisone, corticosterone, Thorotrast, and trypan blue. The precise mode through which these materials function is not clearly understood, although many suggestions have been made concerning their probable role.[30-34]

It is conceivable that other substances or other means will prove to be even more effective blocking agents than those heretofore used; hence, further experimentation along these lines is in process.

Summary

(1) HEp 3 can be transferred for 2 to 8 generations in C3H/JAX, C57BL/10, BALB/c, and Swiss mice.

(2) The best results were achieved when the tumor was transplanted by intramuscular inoculation and the mice were treated with X irradiation of 100 r and injected with 3.5 to 4.5 mg. cortisone.

(3) HEp 3 can be transferred successively and continuously to DBA/2 mice.

Acknowledgments

The authors are indebted to Shozo Takayama for assistance in pathological examination, and to Morris N. Teller for supplying the tumor.

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SUCCESSFUL INTRAMUSCULAR GROWTH OF HUMAN TUMOR IN CONDITIONED MICE*

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INTRODUCTION

The classic experiment of heterologous transplantation of human tumor, which was an attempt to transfer cancer from man to dog, was recorded by Peyrilhe in 1775.[1]

In 1840 Langenbeck injected the vein of a dog with the juice from a human medullary carcinoma of the humerus. Two months later several round nodules were found in the lung. However, Rudolf Virchow, the pathologist, found the structure more similar to a spontaneous cancer in the dog than to that in the human.[1]

In 1884 Zahn[2] transplanted a human enchondroma to the anterior chamber of the eye of a rabbit, but failed to find evidence of its growth.

Klebs[1] in 1890 examined fragments of human carcinoma removed from the peritoneal cavities of white rats at varying intervals after introduction. He found that epithelial constituents of the graft had vanished by the third day.

In 1912 Murphy[3, 4] reported that the Jensen rat sarcoma grew serially in the chick embryo; however, he did not succeed in propagating the tumor in chickens.

In 1938 Greene[5-7] reported that human uterine adenoma and adenocarcinoma could be maintained in the rabbit. This tumor was serially transferred for several generations.

In 1951 a new approach to the problem of heterologous transplantation of human cancer was described by Toolan, who later reported the survival and proliferation of 90 per cent of 101 human tumors in rats and hamsters treated with cortisone and/or X irradiation.[8-10]

Recently many experiments dealing with heterologous transplantation of human tumor have been reported by Sommers *et al.*,[11] Hoch-Ligeti and Hsue,[12] and Patterson *et al.*[13]

The experiments reported in the first part of this paper were set up in the hope of developing human tumors transplantable to mice. In this work the mice were treated with cortisone, X irradiation, and Thorotrast,‡ either singly or in various combinations.

The second part of the paper follows histologically the development of a human tumor from one day to one week after transplantation.

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‡ A proprietary colloidal suspension of thorium dioxide.

METHODS AND MATERIALS

The Swiss albino mice* and mice of the BALB/c and DBA/2 strains† used in the first part of this experiment were all females weighing approximately 12 to 15 gm. The human tumors, HEp 3 and HS 1, used for this study were maintained in our laboratory as transplantable tumors in albino rats treated with X irradiation and cortisone acetate.‡

Nine or 10 days after transplantation into rats, the tumors were aseptically removed and an 80 per cent tumor-tissue suspension was prepared by the same technique described previously by Toolan. About 0.5 cc. of suspended tumor was implanted intramuscularly in the right thigh or, subcutaneously, in the right flank of the mouse.

In order to determine the combination of strain and treatment that would yield the most successful transplantation, the experiment was carried out in the following manner. Forty-two groups of 5 mice each were used in 6 kinds of treatment: cortisone only, Thorotrast only, X irradiation only, cortisone plus Thorotrast, X irradiation plus Thorotrast, and cortisone plus X irradiation. In the treatment using cortisone, immediately after transplantation 1 injection was given subcutaneously near the nape of the neck at a dose of 100 mg./kg. of body weight, and 2 others were administered on alternate days at a dose of 66 mg./kg., making a total of 3 injections. The Thorotrast was injected intraperitoneally immediately after transplantation at a dose of 2500 mg./kg. of body weight and twice again on alternate days at a dose of 1660 mg./kg., making a total of 3 injections. In the treatment using X irradiation, each mouse received 1 dose of 100 r total body irradiation from 1 to 3 days prior to implantation of tumor.

The animals were sacrificed between 8 and 10 days after transplantation. Microscopic examination was made of the samples taken at the time of transplantation. Tumor tissue was immediately fixed in Zenker's or Vandergrifft's solution and cut at 5 μ. Most of the tissue was stained with hematoxylin and eosin or with Mallory stain.

In the second part of this experiment, female Swiss albino mice weighing approximately 12 to 15 gm. were used in 2 groups of 10 mice each. One group received X irradiation and cortisone acetate; the other group was untreated. The tumor used here was the same HEp 3 used in the first part. About 0.3 cc. of suspended tumor prepared as previously described was implanted subcutaneously in the right flank of the mouse. Immediately after tumor transplantation the mice were injected subcutaneously near the nape of the neck with cortisone acetate at a dose of 100 mg./kg. of body weight and they were injected again on alternate days at a dose of 66 mg./kg., making a total of 3 injections.

The animals were sacrificed between the first and the seventh days after transplantation. Each tumor was removed with skin from the animal, then immediately fixed in Vandergrifft's solution and cut at 5 μ. Most of the tissue was stained with hematoxylin and eosin or with Mallory stain, and the metachromasia were stained with toluidine blue at *p*H 6.2.

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* Swiss albino mice were obtained from Taconic Farms, Germantown, N. Y.

† The BALB/c and DBA/2 strains were obtained from the Roscoe B. Jackson Memorial Laboratory, Bar Harbor, Me.

‡ Cortisone acetate in the form of Cortone was supplied by Merck Sharp & Dohme, Philadelphia, Pa.

Results

Part 1

Swiss strain, HEp 3 tumor tissue.

(1) Treated with cortisone only. The transplanted tumors reached 1.5 × 1.8 cm. in diameter and weighed approximately 0.8 gm. 8 days after intramuscular transplantation. A small noninfected cyst formation was observed in the center of the tumors. Microscopically, the peripheral part of the tumor revealed a picture closely resembling the original tumor. In this test the tumors could be maintained only up to the third generation.

Through the third generation there was no infiltration of polymorphonuclear leukocytes, lymphocytes, or plasma cells in the specimens examined. In the second generation the tumor cells were observed to have invaded the small artery walls, and in the fourth generation a marked polymorphonuclear leukocytic infiltration was observed in the transplants.

(2) Treated with X irradiation only. This group received tumor tissue as subcutaneous transplants. Tumor growth was poor in all animals. At the end of 8 days no suitable material was available for transfer to the next generation.

(3) X irradiation plus Thorotrast. Two days after transplantation, all animals died, probably due to treatment. It was concluded that the treatment was too intense for weanling mice.

(4) Thorotrast only. In the first generation the tumor reached about 1.0 × 1.2 cm. in size 8 days after intramuscular transplantation. Microscopically, in the first generation, pyknotic and sometimes vacuolar degenerating cells were encountered between the muscle and tumor tissue. The transplants of the second generation showed complete necrosis. No viable tumor cells were observed.

(5) Cortisone plus Thorotrast. This is one of the best treatments for securing good tumor growth and one that made possible permanent transplantation into conditioned heterologous hosts. In every generation the tumor was removed 8 days after intramuscular transplantation, at which time it was about 1.8 × 1.5 cm. in diameter; it was rubbery, elastic, and well vascularized, and it displayed no cyst formation. The tumor tissue in this series is now being maintained in the twelfth transplant generation. The general cellular morphology was similar to that of the original tumor. No lymphocyte or leukocyte infiltration was seen. Mitotic figures were prominent.

(6) X irradiation plus cortisone. This series was maintained up to the fourth and fifth transplant generations. When the tumor was transplanted into the subcutaneous tissue in this series, it did not grow as judged macroscopically. When the tumor was transplanted intramuscularly, from the first to the fourth generations, the transplanted tumor cells were very similar

microscopically to the original HEp 3 cells. In all generations the tumor cells had a tendency to infiltrate the muscle, but there was no infiltration of lymphocytes, plasma cells, or polymorphonuclear leukocytes. In the fifth generation, however, more than half of the tumor transplant was replaced by necrotic tissue, and many polymorphonuclear leukocytes were seen between the necrotic part and the still viable tumor tissue—tissue in which mitotic figures were seen.

Swiss strain, HS 1 tumor tissue. Macroscopically there was no growth of tumor in any of the treated groups. The tumor tissue that was transplanted intramuscularly to animals treated with cortisone only, Thorotrast only, and cortisone plus Thorotrast showed similar histological patterns. The implanted tumor tissue showed two layers, the outer layer and the inner layer. The former was characterized by degenerating tumor cells and the latter by necrosis.

On the other hand, the groups treated with X irradiation only, X irradiation plus Thorotrast, and X irradiation plus cortisone revealed complete disappearance of tumor cells, the transplants being entirely replaced by fibrous tissue.

BALB/c strain, HEp 3 tumor tissue. Macroscopically there were no recognizable tumor formations in the host when it was treated with cortisone only, X irradiation only, Thorotrast only, or cortisone plus X irradiation.

In this series, one of the most effective treatments was cortisone plus Thorotrast. The tumors of this series showed a histological pattern similar to that of the original tumor. The cells were of variable size with abundant cytoplasm, prominent nuclei, and active mitotic figures. Unfortunately, however, this transplantable tumor was lost in the eighth generation. At this time lymphocyte infiltration was observed between the tumor and the muscle. On the other hand, the groups receiving cortisone only, X irradiation plus Thorotrast, or X irradiation plus cortisone revealed uniform histological patterns. Upon gross examination, no growth of the tumor was apparent; however, a microscopic examination revealed active mitosis of the tumor cells, several of which infiltrated the surrounding muscle.

BALB/c, HS 1 tumor tissue. In this series, also, noninfected cysts were

TABLE 1

RESULTS OF TRANSPLANTATION INTO VARIOUS STRAINS OF CONDITIONED MICE*

Strain	Tumor	No treatment of host	Cortisone	X ray	Thorotrast	X ray plus Thorotrast	Cortisone plus Thorotrast	X ray plus cortisone
Swiss	HEp 3	−	+	±	±	−	+	+
	HS 1	−	−	−	−	−	−	−
BALB/c	HEp 3	−	±	±	−	−	+	±
	HS 1	−	−	−	−	−	−	−
DBA/2	HEp 3	−	±	±	±	±	±	+
	HS 1	−	−	−	−	−	−	−

* The + represents successful transplantation, the − indicates that no viable tumor tissue was present, and the ± indicates that there were tumor cells remaining, but there was no active growth.

formed in all treatment groups. Histologically, the tumor belonged to the nonreactive type.

DBA/2, HEp 3 tumor tissue. The best treatment found for this series was X irradiation plus cortisone. One tumor was carried through to the twentieth generation before it was discontinued. This tumor was regularly implanted into the muscle.

Microscopically, the tumor showed the characteristics of the original tumor, which was abundantly supplied with thin-walled blood vessels. No inflammatory reaction was observed in any generation. On the other hand, there was no evidence of macroscopic tumor formation in the groups treated with cortisone only, X irradiation only, Thorotrast only, X irradiation plus Thorotrast, or cortisone plus Thorotrast. However, survival of the tumor tissue was observed microscopically. The characteristics of this tissue were similar to those of the parent tumor, but the mitotic figures were not so extensive. These tumor cells were surrounded by fibrous tissue, and marked inflammatory cell infiltration was observed between the tumor and adjacent muscle.

DBA/2, HS 1 tumor tissue. HS 1 did not grow in any of these treated hosts, either. Macroscopically, there were some noninfected cysts that were thinly encapsulated. Histologically, there was no evidence of infiltration by the transplanted tumor cells.

Part 2

Treated group.

(1) Twenty-four hours after transplantation. This animal was treated with 100 r X irradiation plus cortisone acetate at the rate of 100 mg./kg. of body weight. The tumor reached about 0.8 × 0.7 cm. in size and was thinly encapsulated, but no noticeable blood vessel formation was observed around the tumor graft. The tumor graft revealed 2 layers histologically, an outer and an inner layer. The tumor cells in the inner layer became necrobiotic and were characterized by pyknotic nuclei, vacuolar degeneration of cytoplasm, and cell debris. A few viable tumor cells remained. On the other hand, in the outer layer the tumor cells were more varied in size, the nucleus was hyperchromatic, and nucleoli were apt to be more prominent.

(2) Two days after transplantation. This animal was treated in the same manner as was No. 1. Histologically, the border between the inner and outer layers became more clearly divided. The inner layer showed a more advanced necrobiosis while, in the outer layer, the tumor cells revealed more active growth characterized by many mitotic figures.

(3) Three days after transplantation. This mouse was treated with 100 r X irradiation plus 166 mg./kg. cortisone acetate. The tumor was 1.0 × 1.0 cm. in size and adhered to the skin. Histologically, the tumor cells had many mitotic figures; in some parts tumor invasion was observed in the subcutis. In this case one of the most dramatic observations was dilatation of the subcutaneous blood vessels.

(4) Four days after transplantation. This mouse also received 100 r X irradiation plus 166 mg./kg. cortisone acetate by the time of autopsy. Mac-

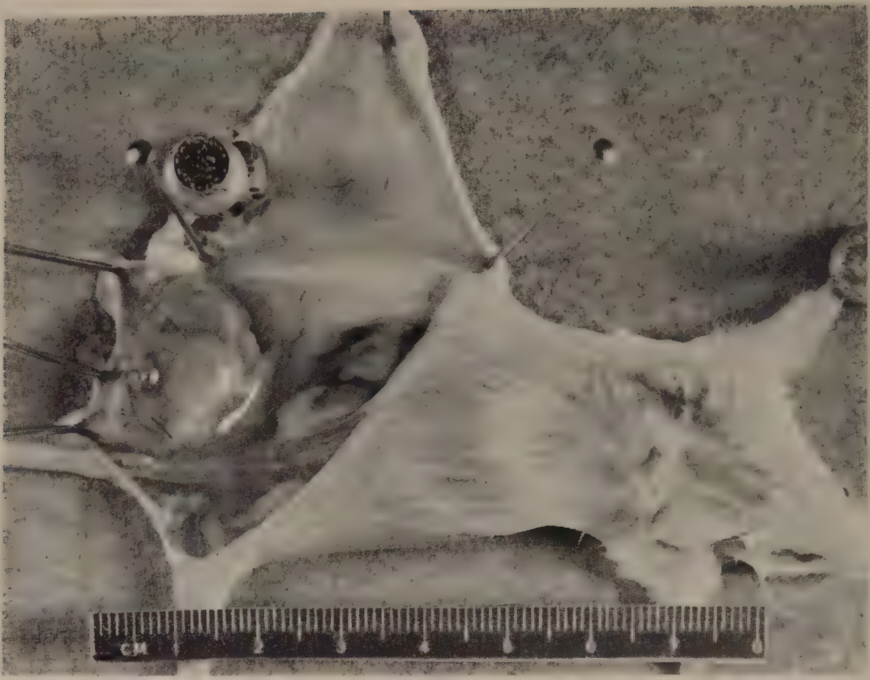

FIGURE 1. HEp 3, sixth generation, 9 days after intramuscular transplantation to Swiss strain mouse treated with Thorotrast plus cortisone.

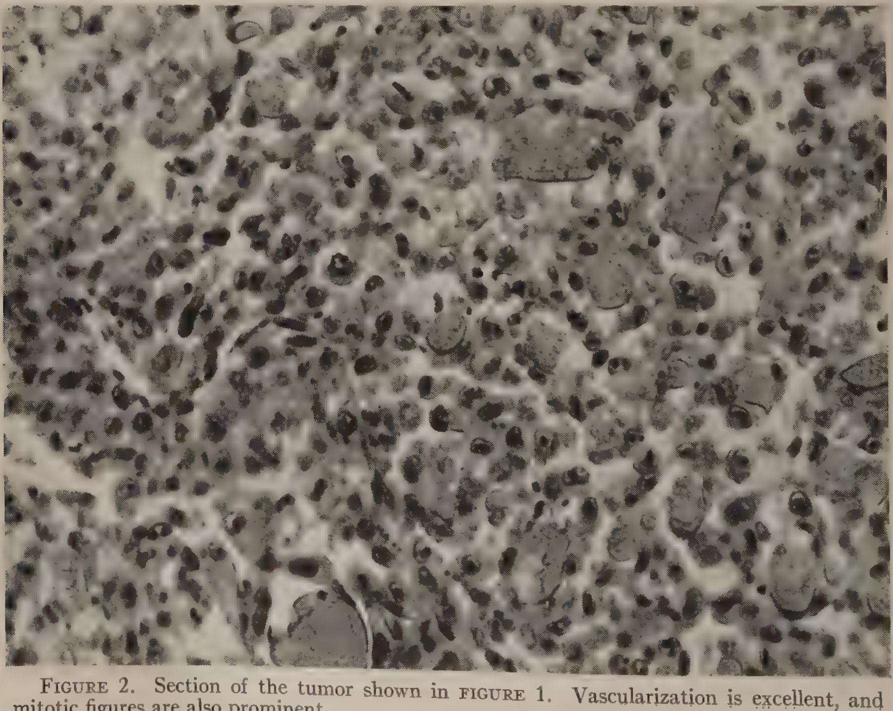

FIGURE 2. Section of the tumor shown in FIGURE 1. Vascularization is excellent, and mitotic figures are also prominent.

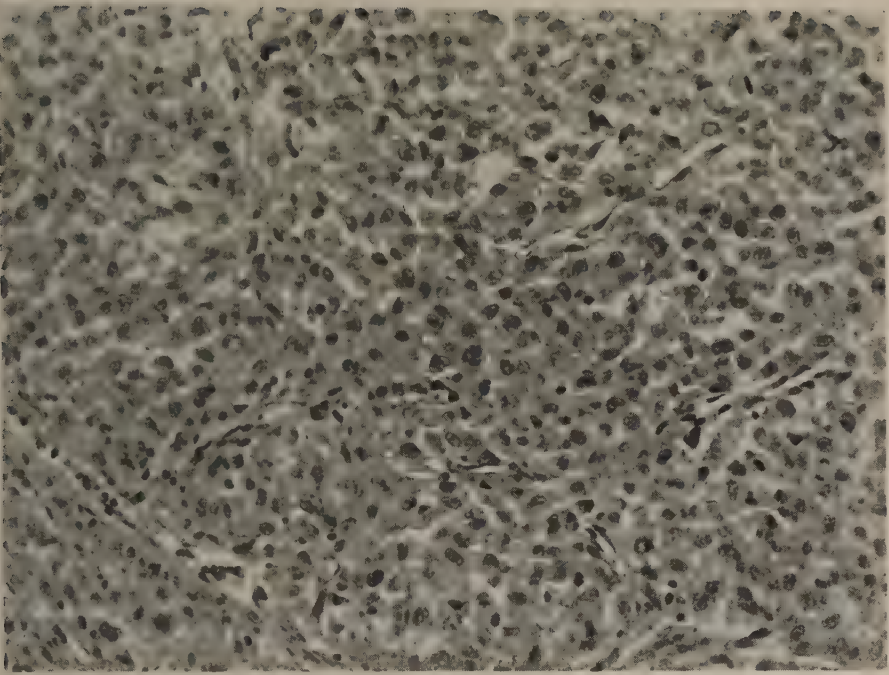

FIGURE 3. HEp 3, sixth generation, 8 days after intramuscular transplantation to a BALB/c strain mouse treated with cortisone plus Thorotrast. General morphology is similar to the original tumor.

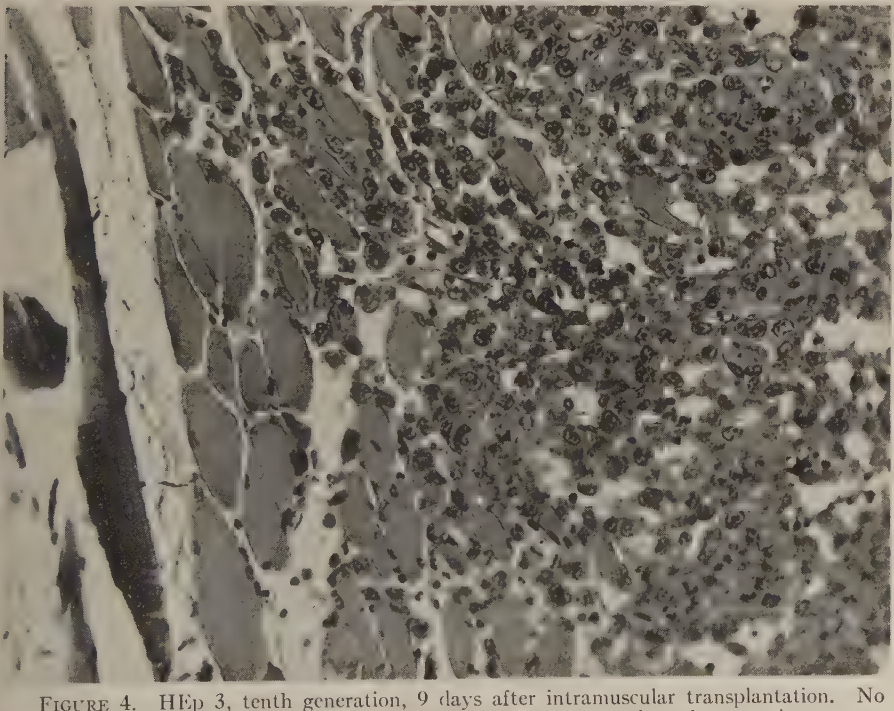

FIGURE 4. HEp 3, tenth generation, 9 days after intramuscular transplantation. No lymphocyte and plasma cell infiltration is seen between the muscle and tumor tissue—non-round cell infiltration type.

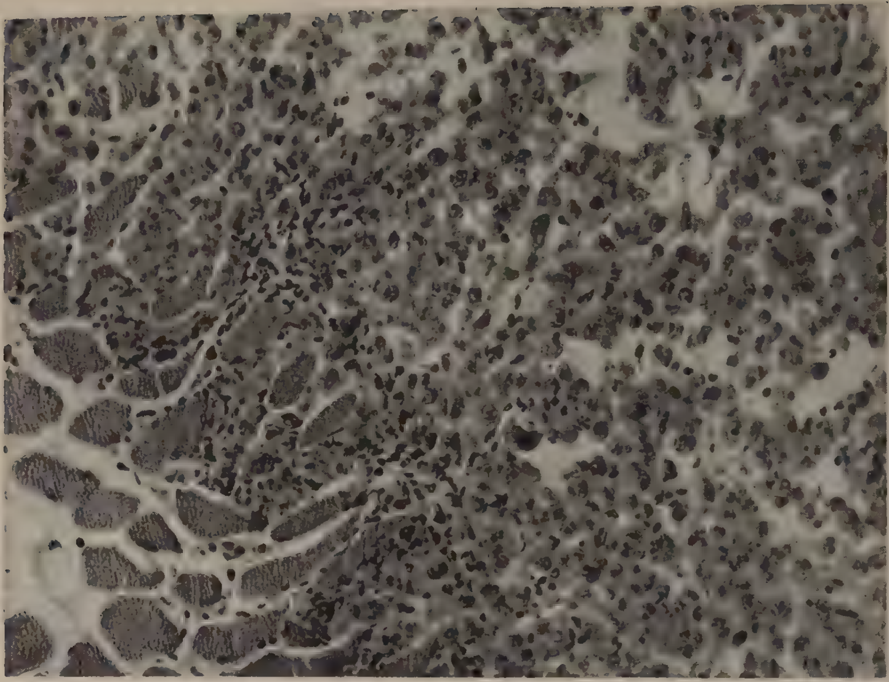

FIGURE 5. HEp 3, fifth generation, 9 days after intramuscular transplantation to a Swiss strain mouse treated with both cortisone and X irradiation. Lymphocyte and plasma cell infiltration is seen between the muscle and tumor tissue—round cell infiltration type.

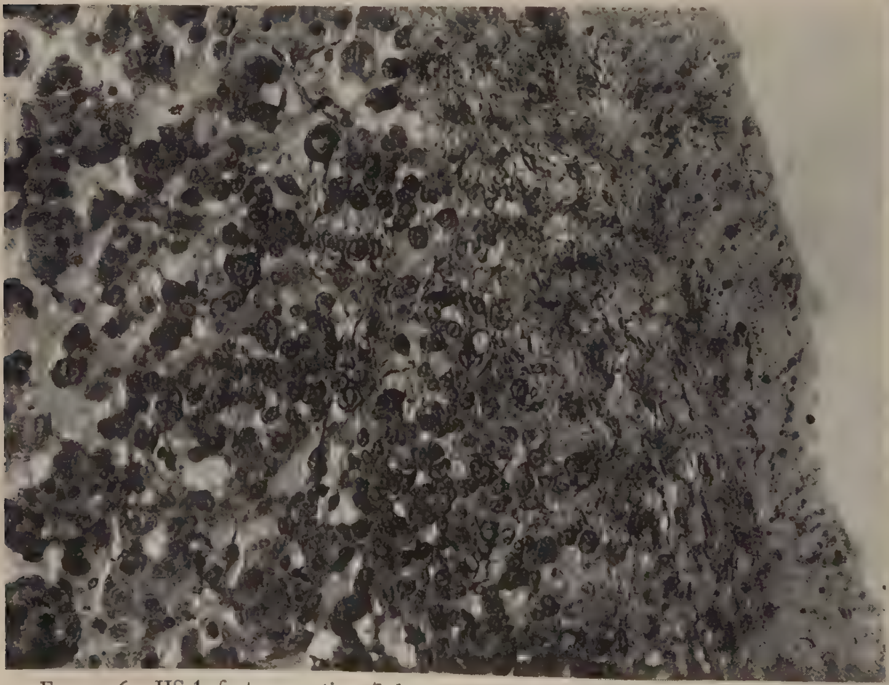

FIGURE 6. HS 1, first generation, 7 days after intramuscular transplantation to a Swiss strain mouse treated with cortisone and X irradiation. The transplanted tumor tissue is necrobiotic—nonreactive type.

roscopically, the tumor was the same as that of No. 3; moreover, it showed invasion into the cutis, microscopically.

(5) Five days after transplantation. This mouse received 100 r X irradiation plus 233 mg./kg. cortisone acetate. The tumor also showed severe adhesion to the skin; it was 1.1 × 1.1 cm. in size and was rubbery in consistency. The outer surface was white-gray and uniform in composition. Histologically, the necrotic part was located in the center, but was scanty in amount while, in the outer layer, the tumor cells had a large amount of cytoplasm and prominent nuclei. Mitotic figures definitely were encountered more frequently within this specimen.

(6) Six days after transplantation. The mouse in this case was conditioned in the same manner as was No. 5. The tumor was about 1.2 × 1.0 cm. in size and elastic and rubbery in consistency. The tumor mass was characterized by a large amount of cytoplasm, acidophilic nuclei, and fairly frequent mitotic figures. Vascularization in the tumor was excellent. In the center of the tumor, a small amount of necrotic tissue still remained.

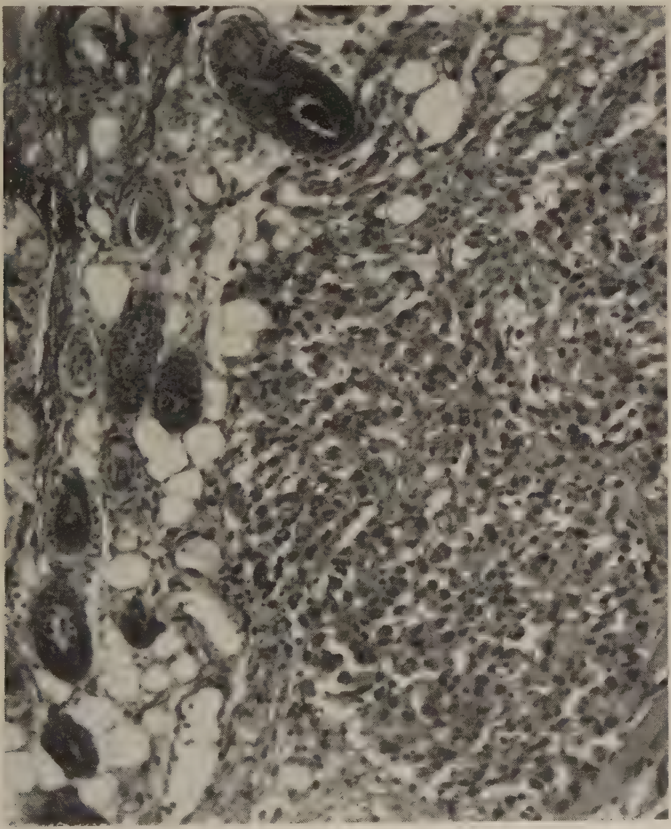

FIGURE 7. Four days after transplantation of treated group. The tumor has invaded the subcutis.

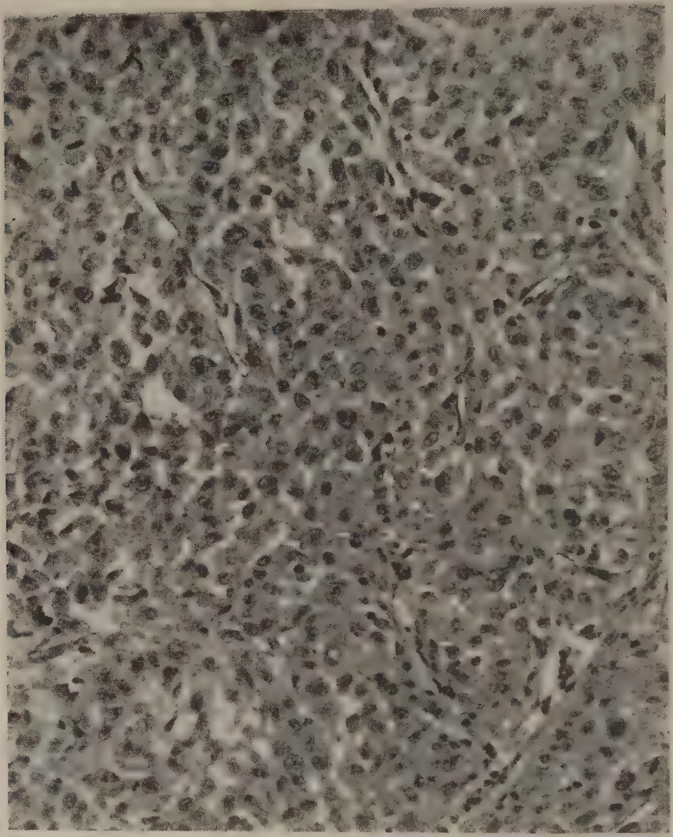

FIGURE 8. Seven days after transplantation of treated group. Vascularization is excellent and mitotic figures are prominent.

(7) One week after transplantation. The treatment was the same as that for No. 5. The tumor showed the same histological pattern as that of No. 6

Nontreated group.

(1) Twenty-four hours after transplantation. The tumor was an ovoid soft mass 0.5 cm. in diameter. Histologically, the transplant showed 3 layers. In addition, there were many lymphocytes, plasma cells, and a few leukocytes in this specimen. Specifically, unlike the series in the treated group, there was no atrophy of the epidermis and accessory organs of the skin.

(2) Two days after transplantation. The size of the transplant was the same as that of No. 1. The transplant showed 3 layers, that is, the outer necrobiotic layer, the inner necrotic layer, and the middle layer. In the middle layer viable tumor cells were still encountered.

(3) Three days after transplantation. The tumor was a small, rather firm mass. In contrast to the previous case, fibrous tissue instead of necrotic tissue

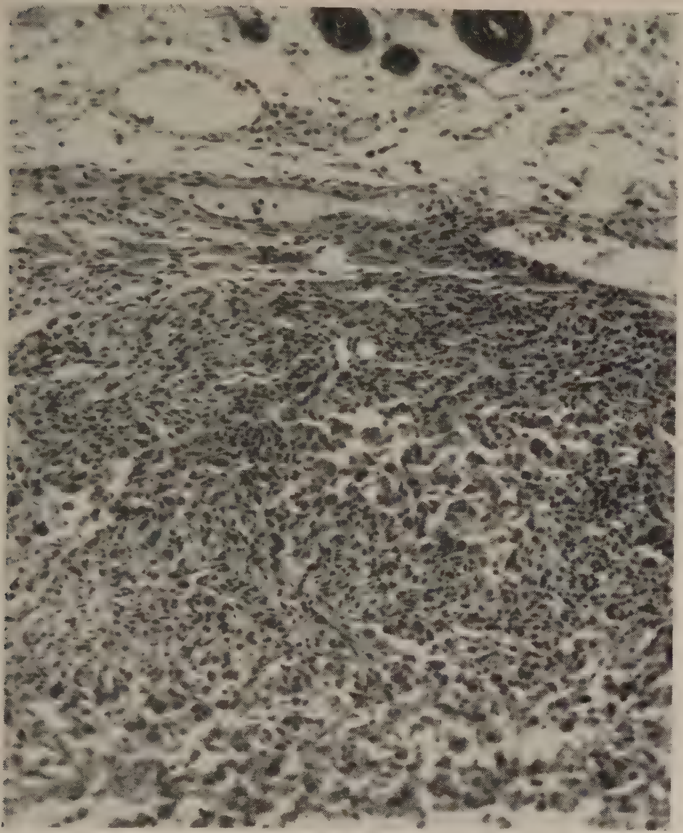

FIGURE 9. Four days after transplantation of nontreated group. Degenerating tumor cells still remain in the middle layer.

was seen in the outer layer. In the middle layer transformed tumor cells still remained. The inner layer also contained necrobiotic tumor cells and cell debris.

(4) Four days after transplantation. This case was macroscopically and microscopically similar to No. 3. The outer layer had a greater fibroplastic tendency than did the outer layer of No. 3.

(5) Five days after transplantation. The outstanding observation was the complete disappearance of the tumor cells from the middle layer. The outer layer was entirely replaced by fibrous tissue. The transition between the fibrous tissue of the outer and middle layers was obvious.

(6) Six days after transplantation. The microscopic findings were the same as those of No. 5.

(7) One week after transplantation. The tumor graft was completely replaced by fibrous tissue. No tumor cells were observed.

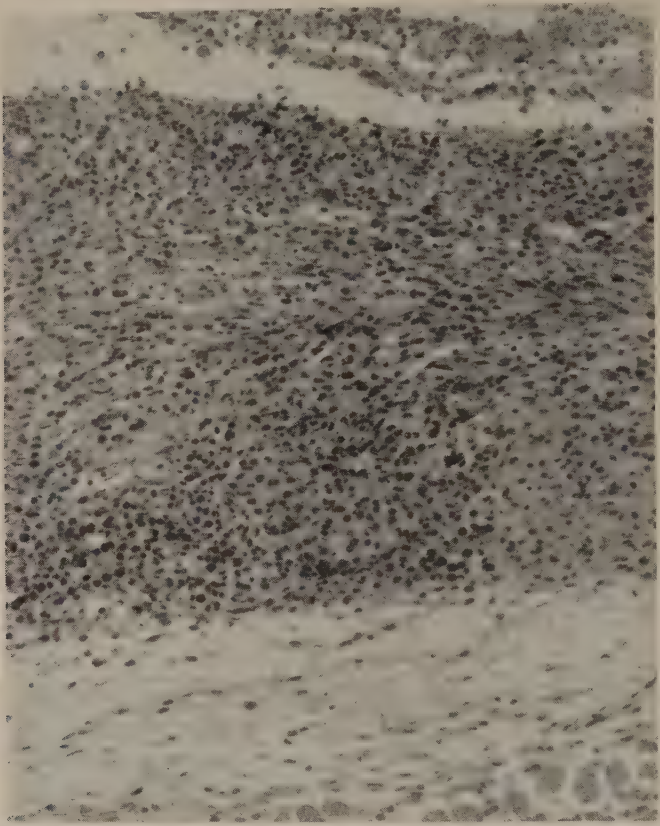

FIGURE 10. Seven days after transplantation of nontreated group. The tumor graft has been completely replaced by fibrous tissue.

DISCUSSION

We have no definite knowledge of the biological response of the host when it is treated with cortisone, X irradiation, and Thorotrast.

In the present study, Toolan's method of transplantation of human tumor into rats conditioned by cortisone, X irradiation, Thorotrast, or a combination of these was employed.

In this experiment, there was no strain of mice in which subcutaneous transplantation of either HS 1 or HEp 3 was successfully performed. When the tumors were implanted in the muscle of the mouse, the transplantation was successful in all but HS 1. This difference in transplantability in 2 sites does not appear in the case of homotransplantation. The reason for the intramuscular transplantation's yielding good results while the subcutaneous transplantation was negative is not clear from the present experiment. In 1952, Talalay *et al.*[14] discussed the implantation site using the Walker tumor and reported that slower growth was obtained in the subcutaneous site than in the intramuscular or intraperitoneal sites. However, no definite conclusions were drawn.

Morphologically, there are three reaction types in the transplants: one with round cell infiltration, one without round cell infiltration, and a nonreactive type. The first type has lymphocyte or plasma cell infiltration between muscle and tumor tissue. The second has no such cell infiltration. Those tumors that show no lymphocyte or plasma cell infiltration can be transplanted continuously. When HS 1, a nonreactive type of tumor, is transplanted, it shows no growth, and the implanted tissue soon becomes necrotic.

Tumors transplanted to conditioned animals retain the human tumor pattern both serologically and biochemically.[15]

The histological findings of the second part of our work were similar to those of the homotransplantation of DAB (4-dimethylaminoazobenzene) hepatoma, which was reported in 1941 by Satomi.[16]

In the present experiment the developmental transformation of tumor tissue that was transplanted to conditioned mice was readily visible. We therefore believe that this comparative experiment showing the relation of time to tumor growth will contribute to chemotherapeutic experiments using transplantable human tumors.

It is our belief that the terms "tumor survival" and "successful transplantation" must be used with two clearly separate meanings. Tumor survival should refer only to tumor cells remaining in implanted tissue that shows no sign of active growth, for example, neither active mitosis nor increase in number of tumor cells. On the other hand, successful transplantation should refer to tumor tissue that has active growing signs and usually will kill the host.

Confusion arises also because the term "transplantation" is used to refer both to the technique of inoculation or transportation and to the action of the tumor cells after the transportation or inoculation.

It should also be borne in mind that although a transplanted human tumor may be growing when transplanted into conditioned animals, eventually the transplanted tumor tissue will show regression, either with or without conditioning.

It is also peculiar that in heterotransplantation of human tumors there are generally no metastases in the distant organs of the mouse. However, previous to these experiments we had had a chance to observe a case of liver metastasis in a mouse that had received a human tumor transplant in the muscle. Metastatic nodules located in the portal area revealed three or four groups that consisted of a few tumor cells. Histologically, this metastasis probably occurred hematogenously. In our present experiments there are no metastases in any organs.

Summary

Part 1

The combination of mouse strain and host conditioning treatment that would yield successful transplantation was investigated.

The HEp 3 tumor has been maintained intramuscularly in Swiss strain mice treated with cortisone plus Thorotrast for more than 12 generations, but it could not be maintained beyond the fifth generation when the mice were treated with X irradiation plus cortisone.

The best method of conditioning in BALB/c strain mice was by the use of cortisone plus Thorotrast. With this combination the tumor was maintained intramuscularly until the eighth generation.

In DBA/2 strain mice treated with X irradiation plus cortisone, HEp 3 has been maintained intramuscularly for more than 20 generations.

HS 1 did not grow in any strain of mice, regardless of conditioning.

Part 2

A comparative study of HEp 3 transplanted to groups of Swiss strain mice, some treated with X irradiation plus cortisone and others not treated, was made. The animals were sacrificed on the first to the seventh days after transplantation.

Marked histological evidence of growth of the implanted tissue was noticed first on the third day after transplantation in the treated animals.

Grossly, the tumor formation was recognized 4 or 5 days after transplantation.

On the other hand, the transplanted tumor tissue in the nontreated group showed regression 2 or 3 days after transplantation. Five days after transplantation the tumor tissue was partially replaced by fibrous tissue and only degenerating tumor cells were observed. One week after transplantation the transplanted tumor tissue was completely replaced by fibrous tissue.

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Discussion of the Paper

Comment: I do not know whether X irradiation is the absolute essential for the conditioning of animals, since cortisone has worked well in some instances. However, as a plea I should like to indicate that, for most people who are about to go into conditioning of animals, it may be helpful to know not only the radiation of r, which is a variable figure, but also the half-layer value (HLV). When we set out to condition animals and tell the radiologist that we require 100 r, he asks what kind of 100 r we want. He must know the HLV for copper and aluminum. Thus, for all those who work with mice, hamsters, goats, chickens, or pigs it might prove very helpful if, in addition to stating the number of r, we provide the HVL.

HOST CONDITIONING IN EXPERIMENTAL CHEMOTHERAPY*

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The screening of potential anticancer agents against transplantable human tumors in mammals would be virtually nonexistent without procedures for altering the species resistance of animals to heterologous tissues. Whatever information of clinical value results from the study of transplantable human tumors will probably stem directly from the fact that host conditioning has made possible the extensive study of such tumors. It is sometimes necessary to emphasize this thought in order that the questions raised by discussing variations in conditioning procedures will not obscure the real reason for using a heterologous tumor-host system for experimental chemotherapy, that is, that this tissue was derived from human patients, has retained human characteristics,[1, 2] and may be more like the human neoplasm in its response to chemical compounds than animal tumors.

The development of a test system using transplantable human tumors involves the same principles and procedures important in the development of any tumor-host test system.[3] Moreover, as with any tumor-host system, confidence in the reliability of test results depends on an awareness and consideration of the influence on these results of variables operating within that system.

The atypical variable encountered in assaying human tumors for response to potential anticancer agents is the resistance of mammalian species to grafts of heterologous tissue and the effect on both tumor and host of the agents used to lower that resistance. The objective in conditioning for experimental chemotherapy of transplantable human tumors is to use the least amount of conditioning consistent with maximum growth of the transplant and minimum effect on the host.

The agents primarily used for host conditioning are X irradiation—generally 150 r—and/or either cortisone or hydrocortisone. These corticoids possess many metabolic activities in addition to their ability to depress the lymphoid organs and cells important to the defenses of the host to foreign tissue. In rats receiving doses of cortisone of a magnitude needed for effective conditioning for human tumor growth, interference with metabolism is evidenced by the inability of the animals to gain weight normally and by a reduced tolerance to various chemicals (P. C. Merker, M. N. Teller and J. E. Palm, unpublished data; see also Teller *et al.*, "The Human Tumor in Cancer Chemotherapy in the Conditioned Rat," elsewhere in this monograph). The extent to which the corticoids either directly, by acting with test drugs, or indirectly, by side effects on metabolism, influence experimental chemotherapy results is not

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known, and probably cannot be definitely known until nondebilitating, noncorticoid conditioning agents are found. Nonsteroid agents as effective as the corticoids for conditioning of the host have not been reported.

The experiments reported here represent part of an effort to determine the extent to which the bioassay for experimental chemotherapy of human tumors might be improved by varying the dosage and time of injection of various corticoids for host conditioning.

Materials and Methods

Two human tumors of the group established in heterologous hosts by H. Toolan have been used. They are the human sarcoma, HS 1, and the human epidermoid carcinoma, HEp 3, which have been described previously.[4] Transplantation procedures of this laboratory have been described in detail by Teller *et al.* elsewhere in this monograph. Briefly, postweanling female albino rats weighing between 55 and 63 gm. have received 300 mg. of tumor suspension subcutaneously in the right flank between 1 and 3 days following a single total body dose of 150 r X radiation. Inocula were prepared by mincing tumors and adding a buffered Locke-Ringer salt solution containing the antibiotics penicillin and streptomycin. As a "standard" conditioning procedure, one consisting of 4 injections of cortisone acetate, 60 mg./kg. per injection, beginning on the day of transplantation and continuing on alternate days thereafter, was used. Other host conditioning with steroids is described later in this paper. The steroids used were cortisone acetate,* corticosterone,* and hydrocortisone acetate.† Experiments were terminated on the tenth and twelfth day after implantation for HEp 3 and HS 1, respectively. At this time the rats were weighed and the tumors excised and weighed. In some experiments an external measurement was used. This was the diameter in cm., which was obtained by extracting the cube root of the product of tumor length × width × depth.

Therapy was conducted in the following manner. The same tumor inoculum was transplanted to 24 rats. Twelve rats were conditioned in a single way and 12 with the variation. Each of the 2 groups of 12 rats was divided into a treated and a control group, and the same compound was administered to both treated groups. Thus, some indication of the response of the same tumor in a different conditioning environment could be obtained. Treatment was started 24 hours after transplantation and continued as single intraperitoneal injections administered daily, with the exception of Sundays, for 7 to 8 days. Control animals received daily injections of the corresponding compound diluent, physiological saline, or 0.5 per cent carboxymethylcellulose in physiological saline.

Experimental

Upper and lower limits of conditioning. The effects of doses of cortisone smaller than the "standard" may be seen in TABLE 1. A rapid falling off of

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* Cortisone acetate in the form of Cortone, and corticosterone were supplied by Merck Sharp & Dohme, Philadelphia, Pa.

† Hydrocortisone acetate was supplied by Chas. Pfizer & Co., Inc., Brooklyn, N. Y.

Table 1

Effect of Reducing Host Conditioning on the Growth of HS 1 and HEp 3

Tumor	Host conditioning: 150 r plus			Tumor takes*	Tumor takes (per cent)	Mean tumor size (cm.)
	Corticoid	Number of injections	Amount per injection (mg./kg.)			
HS 1	Cortisone	4	60	20/25	80	2.3
	Cortisone	4	45	22/29	76	2.4
	Cortisone	4	30	12/30	40	1.9
HEp 3	Cortisone	4	60	19/19	100	2.3
	Cortisone	4	45	19/19	100	2.2
	Cortisone	4	30	10/20	50	2.2

* A tumor graft was considered a take if the size palpated was larger than that of the original inoculum.

the number of takes as well as a decreased tumor size occurred in rats bearing HS 1 when the amount of cortisone per injection was reduced. At the lowest dose, 30 mg./kg. per injection, only 40 per cent of the animals had tumors on the twelfth day after transplantation. Although the average size of HEp 3 tumors was not smaller at 30 mg./kg. of cortisone, the percentage of takes of this tumor at this lowest dose was only 50 per cent, indicating insufficient conditioning to overcome host resistance to the tumor. For both tumors, reducing the amount of cortisone for host conditioning by one fourth or one half of the standard resulted in reduced tumor takes and/or tumor size. The effect of reducing the number of cortisone injections is shown in TABLE 2. Under the 3-injection schedule (FIGURE 1 shows the relationship of 3 and 4 injections, respectively, to transplantation and therapy), the 2 tumors differed as to the amount of cortisone per injection required for best tumor growth. For growth of HS 1 comparable to the control it was necessary to increase the amount of cortisone per injection from 60 to 80 mg./kg. When this was done, similar results were obtained under the 2 injection procedures. Although there were indications of decreased host mortality and some host

Table 2

Effect of Different Cortisone Schedules on Growth of HEp 3 and HS 1 in the Rat

Tumor	Conditioning: 150 r plus			Tumor takes	Mean tumor weight (gm.)	Host mortality	Mean host weight change (gm.)
	Corticoid	Number of injections	Dose (mg./kg.)				
HS 1*	Cortisone	4	60	22/22	6.8 ± 2.5	2/24	+6
	Cortisone	3	80	24/24	7.9 ± 5.1	0/24	+11
HEp 3†	Cortisone	4	60	46/46	4.3 ± 1.9	7/46	+4
	Cortisone	3	60	45/45	5.2 ± 1.8	6/46	+4

* Pooled data from 4 experiments.
† Pooled data from 8 experiments.

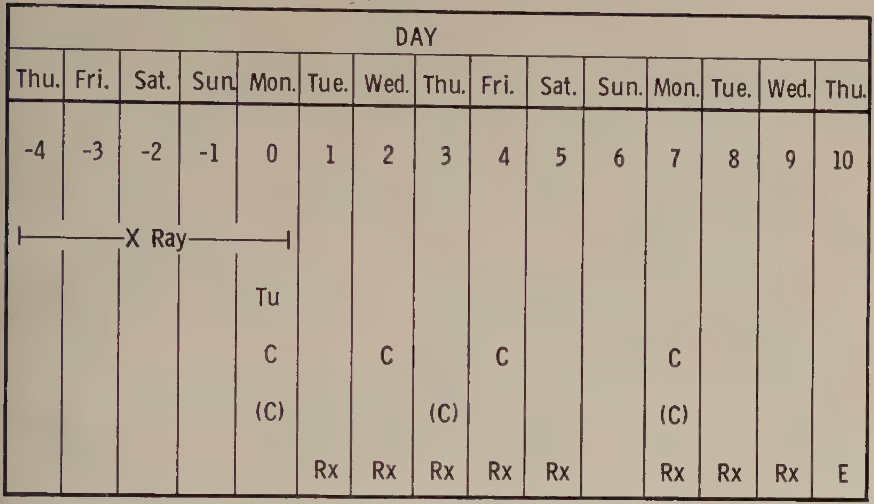
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FIGURE 1. Relationship of cortisone injections to tumor implantation and therapy trials. C represents cortisone acetate procedure (4 injections); (C), variation of regular procedure (3 injections); Tu, tumor; Rx, therapy; and E, evaluation.

weight gain under the 3-injection schedule, there was a greater variation of tumor weight.

Three injections of cortisone at 60 mg./kg. were found, on the contrary, to be sufficient for good growth of HEp 3. This reduced amount of cortisone, in fact, resulted in an average tumor size larger than was found in rats receiving 4 injections of 60 mg./kg. This trend to larger tumor size under a 3-injection schedule was consistent in each of 8 experiments, and an analysis of variance of the data indicated that the difference between the mean tumor weights

TABLE 3

EFFECT OF FIVE COMPOUNDS ON THE GROWTH OF HEp 3 IN RATS FOLLOWING TWO PROCEDURES OF HOST CONDITIONING WITH CORTISONE

Compound	Dose (mg./kg./day)	Results									
		Rats conditioned with 4 injections cortisone (60 mg./kg./injection)					Rats conditioned by 3 injections of cortisone (60 mg./kg./injection)				
		Host mortality		Host weight change (gm.)		Percentage inhibition	Host mortality		Host weight change (gm.)		Percentage inhibition
		T	C	T	C		T	C	T	C	
Thio-TEPA......	2.0	1/6	1/6	−10	−1	26	0/6	3/6	−1	+9	33
Urethan	500.0	0/6	0/6	−2	+9	22	0/6	0/6	−1	+2	12
Azaserine........	2.6	2/6	0/6	−10	+7	56	0/6	0/6	−5	+2	33
6-Mercaptopurine.	30.0	3/6	0/6	−14	+4	43	4/6	0/6	−1	+3	28
Benzimidazole....	250.0	1/6	0/6	+4	+7	41	2/6	0/6	+8	+2	6

following the different treatments was significant ($P < 0.01$). This implied that excess cortisone could have an adverse effect on the growth of HEp 3. A similar effect of cortisone on the growth of HEp 3 in the chick embryo has been reported by J. J. Harris and G. W. Woolley elsewhere in this monograph.

The effect of the 2 cortisone-injection schedules—4 versus 3 injections of 60 mg./kg.—on experimental therapy results is illustrated in TABLE 3. No attempt to assess the antitumor activity of the 5 individual drugs can be made on the basis of the single runs listed. With a single exception, however, the percentage of inhibitions obtained was larger in the groups receiving the greater amount of cortisone, which suggests that the HEp 3 tumor under an adverse conditioning environment may be more sensitive to slight inhibitory effects of the compounds administered.

Effectiveness of other corticoids for conditioning. Hydrocortisone, the predominant free-circulating corticoid found in humans, is structurally very similar to cortisone (FIGURE 2), and the 2 compounds may be used interchangeably for conditioning rats and hamsters for human tumor growth. A comparison of the effectiveness of hydrocortisone and cortisone for conditioning for the growth of HS 1 and HEp 3 under these experimental conditions is presented in TABLE 4. The growth of HEp 3 was very similar in each of the 2 groups. The HS 1 data indicated a slightly lower percentage of successful implants and a lower average tumor weight in rats receiving hydrocortisone treatment.

The predominant, naturally occurring corticoid of the rat[5] is corticosterone, which differs structurally from cortisone or hydrocortisone in that it is not oxygenated at the twentieth carbon atom. Its known metabolic activities are similar to those of hydrocortisone and cortisone, although it has been reported to be less effective in blocking various manifestations of the immune

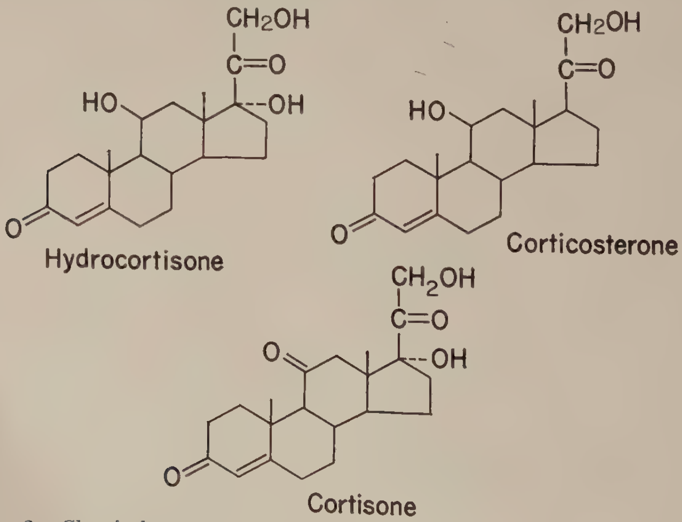

FIGURE 2. Chemical structures of three cortical hormones used for conditioning rats for growth of transplantable human tumors.

TABLE 4

COMPARISON OF CORTISONE AND HYDROCORTISONE AS HOST-CONDITIONING AGENTS FOR GROWTH OF HEP 3 AND HS 1 IN THE RAT

Tumor	Host conditioning: 150 r plus			Takes	Mean tumor weight (gm.)	Host mortality	Host weight change (gm.)
	Corticoid	Number of injections	Amount per injection (mg./kg.)				
HEp 3	Cortisone	3	60	21/21	4.0 ± 1.8	2/21	−1
	Hydrocortisone	3	60	21/21	3.5 ± 1.9	0/21	+4
HS 1	Cortisone	4	60	12/13	5.5 ± 4.5	0/13	0
	Hydrocortisone	4	60	10/13	4.6 ± 3.4	2/13	−1

TABLE 5

COMPARISON OF CORTISONE AND CORTICOSTERONE AS HOST-CONDITIONING AGENTS FOR GROWTH OF HEP 3 IN THE RAT*

Host conditioning: 150 r plus			HEp 3 tumor takes	Mean tumor weight (gm.)	Mortality	Host weight change (gm.)
Corticoid	Number of injections	Amount per injection (mg./kg.)				
Cortisone..........	3	60	41/41	4.0 ± 1.4	4/41	+1
Corticosterone.....	3	120	42/42	4.2 ± 1.2	6/42	+13

* Comparisons represent pooled data from 8 experiments.

processes than either of the other two.[6] TABLE 5 indicates that, when corticosterone was used at a dose of 120 mg./kg. per injection, the growth of HEp 3 was comparable to that occurring when the optimum cortisone-conditioning procedure was employed.

One striking difference between the 2 treatments, however, was observed in a comparison of the weight gain of the hosts. The animals under corticosterone treatment gained an average of 13 gm. during the 10-day experiments as compared to an average 1-gm. increase in weight for the cortisone group. Because of this somewhat lower toxicity of corticosterone to the host, a comparison was made of the effect of several compounds on HEp 3 in rats conditioned by the 2 corticoids, respectively, to see if any interrelationship between toxicity to the host and percentage of inhibition of tumor growth was present. If such were the case, the percentage of inhibition by any compound would be expected to be greater in animals receiving the cortisone treatment, which causes a greater impairment of weight gain than does corticosterone. A survey of the results (TABLE 6) indicated that, in these experiments at least, this did not occur.

An interesting observation is the apparently increased activity of actinomycin D in the animals receiving corticosterone. The data from the 2 individual experiments are presented in TABLE 7. In the first experiment

TABLE 6

EFFECTS OF FOUR COMPOUNDS ON THE GROWTH OF HEp 3 IN RATS FOLLOWING HOST CONDITIONING WITH CORTISONE AND CORTICOSTERONE, RESPECTIVELY

Compounds	Dose (mg./kg./day)	Results									
		Rats conditioned with 150 r + cortisone (3 injections at 60 mg./kg.)					Rats conditioned with 150 r + corticosterone (3 injections at 120 mg./kg.)				
		Host mortality		Host weight change (gm.)		Percentage inhibition	Host mortality		Host weight change (gm.)		Percentage inhibition
		T	C	T	C		T	C	T	C	
Triethylene melamine (TEM)*	0.18	3/12	0/12	−7	+2	0	0/12	1/12	+1	+12	13
Azaserine*	2.6	0/12	1/12	−7	+7	40	1/12	0/12	−3	+18	50
Actinomycin D*	0.06	4/12	3/12	−3	+3	43	2/12	4/12	+3	+11	63
6-Diazo-5-oxonorleucine (DON)	0.05	0/6	0/6	+2	+3	12	1/12	0/12	+3	+12	4

* Pooled data from 2 experiments.

TABLE 7

EFFECT OF ACTINOMYCIN D (0.06 MG./KG./DAY) ON GROWTH OF HEp 3 IN RATS CONDITIONED WITH CORTISONE AND CORTICOSTERONE, RESPECTIVELY

Host Conditioning: 150 r plus	Results						
	Host mortality		Mean host weight change (gm.)		Mean tumor weight (gm.)		Percentage inhibition
	T	C	T	C	T	C	
Experiment 1							
Cortisone	3/6	3/6	−1	+1	3.9	5.6	30
Corticosterone	2/6	4/6	+5	+3	3.5	5.8	40
Experiment 2							
Cortisone	1/6	0/6	−5	+4	1.4	3.8	63*
Corticosterone	0/6	0/6	0	+18	0.7	5.2	86†

* $P < 0.05$.
† $P < 0.01$.

mortality was high, and the tumors of the animals remaining were moderately affected by the actinomycin D. The percentages of inhibitions in the two groups, although slightly greater in the corticosterone-treated rats, were not very different. The second experiment showed a lower mortality, and the tumors in the actinomycin D-treated groups were greatly inhibited. The different degree of inhibition occurring under the two conditioning procedures cannot be explained entirely by the larger size of the control tumors in the corticosterone group, for a comparison of the 2 treated groups showed a significant difference ($P < 0.01$) between the size of the tumors growing in the rats that received cortisone and actinomycin D and those that received corticosterone and actinomycin D, the latter being, on the average, 50 per cent smaller in size.

Comment

Conditioning of mammals with cortisone or other steroids makes possible the extensive study of neoplasms derived from human patients. The problems that host conditioning may contribute to experimental chemotherapy of such tumors seem small in comparison with the impetus to cancer research that the opportunity to study such human tissues should provide. Nevertheless, a real possibility does exist that, in the enthusiasm to test potential anticancer agents against human tumors, ambiguous and/or misleading information will be obtained unless an attempt is made to reduce to a minimum the importance of host-conditioning factors to the experimental design.

The fact that we must use cortisone or similar substances for growth of human tumors should not, over the long term, impede the chances of detecting agents most capable of inhibiting these tumors. Drugs whose activity is controlled or influenced merely by the presence of cortisone in the host should be spotlighted by comparative studies of different human tumors in various host and conditioning environments, including systems such as those of the chick embryo in which no conditioning is required.

The real problem associated with the necessity for host conditioning is the lack of knowledge of the extent to which the results of therapy may in part reflect (1) a greater sensitivity to drugs of tumors in a heterologous environment, (2) residual host resistance, and (3) indirect depressing effects on tumor growth due to the toxicity of the conditioning agents to host metabolism. Superior antitumor agents in these systems will be most readily recognized as such, it seems, when the test system is made as free as possible from spurious antitumor effects caused by less than optimum host conditioning. The host-conditioning procedure of choice for any transplantable tumor should be determined, even as the best procedure for transplantation, the best procedure for therapy, and the best procedures for tumor evaluation must be determined in any tumor bioassay.

The data presented here illustrate an effort to determine the influence of conditioning variations on experimental therapy. Real answers to the questions posed await far more experimentation and possibly the development of nonsteroid conditioning procedures. Two points of some importance to experimental therapy have been demonstrated: (1) the growth of one human tumor may be affected adversely by host-conditioning procedures that are optimum for the growth of a second human tumor, and (2) steroids other than cortisone may be less toxic to the host while allowing tumor growth equal to that resulting from cortisone treatment.

The potential value of transplantable human tumors to clinical therapy cannot be determined until information derived from the study of such tumors is translated into clinical experience. The chances of uncovering and recognizing potentially useful agents will probably be greater when an attempt is made to define the extent to which the heterologous nature of the human tumor graft influences therapy results. With such an approach, it seems unlikely that the factor of host conditioning will limit the discovery of useful information if transplantable human tumors can, as is hoped, yield such information.

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DISCUSSION OF PART VI*

By George W. Woolley

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Historically, we have heard that attempts to transplant human cancer to experimental hosts have been recorded for a century or more: Peyrilhe, 1775; Langenbeck, 1840; Zahn, 1884; and Klebs, 1890.[1] More recently, heterotransplantation has been forwarded by Murphy (in 1912)[2] through transplantation of tissue to the chorioallantois of the chick embryo. The use of X irradiation to reduce resistance to foreign tissue also was explored.[3] More recent work includes that of Greene (1938)[4] and others. In 1951 and later, Toolan,[5] re-explored the use of X irradiation and instituted the use of cortisone in host-conditioning procedures. She achieved successful and continuous implantation of human tumors in conditioned rats and hamsters.[6] Elsewhere in these pages she reviews some of her basic and exciting new findings, placing emphasis on the growth and morphologic potentialities of these tissues in their unusual environment, their capabilities for long-term preservation by freezing, and their stability in retaining their species specificity.

The chemotherapy studies reported from our laboratory are dependent on the methodology of heterotransplantation developed by Toolan. We believe that use of human tissue enhances the probability of picking up, indicating, and evaluating compounds useful to man.

Use of the rat as a host for human tumors has been presented from two laboratories. The basic methodology has been similar. Several hundred agents now have been evaluated, and workable systems of operation are in more-or-less routine use. In our studies, as presented by Teller and discussed by Merker, two human tumors originally introduced by Toolan were the basis for the chemotherapy studies. In this system, young female Wistar rats approximately 4 weeks old (50 to 65 gm.) were X irradiated with a single total body dose of 150 r 1 to 4 days prior to implantation of tissue. Two 180 kvp. tubes were used, one above and one below the animals (not parabolic) at a TSD of 50 cm. (50 r/min.).

For the preparation of inoculum for transplantation, 9- to 11-day-old HEp 3 and 10- to 14-day-old HS 1 tumors were used. Tumors grown subcutaneously in the X-irradiated and cortisone-treated rats were excised, minced with scalpels, and prepared as a suspension in sterile Ringer's salt solution. Each 100 ml. of Ringer's solution contained, in addition, 600 mg. glucose, 20,000 u. penicillin, 60 mg. streptomycin, and 1 ml. of McIlvane's phosphate-citrate buffer (*p*H 7.35 to 7.40). A sample of minced tumor tissue was removed for a test for sterility prior to addition of the diluent.

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For maintenance of tumor supply, 1 cc. of a 70 to 80 per cent tumor suspension was injected subcutaneously into the flank of the X irradiated rat. For chemotherapy trials, one half cc. of a 70 per cent tumor suspension was used. Immediately after implantation of the tissue the rats were injected subcutaneously near the nape of the neck with cortisone acetate* at a dose of 60 mg./kg., and again on alternate days for a total of 4 injections.

All instruments and glassware were sterile, and preparations were carried out in glass-enclosed hoods to maintain aseptic conditions insofar as possible.

Drug therapy was started 24 hours after tumor implantation, and continued as single, daily intraperitoneal injections (Sundays excepted) for at least 8 injections for HS 1- and at least 7 injections for HEp 3-bearing rats. Drug solutions and suspensions were prepared in such concentrations that the dose administered was 0.5 to 0.6 ml. Control animals were injected with the diluent.

Rats bearing HS 1 were sacrificed on the eleventh or twelfth day, and those bearing HEp 3 on the ninth to eleventh day, 24 to 48 hours after the last dose had been given. The tumors were removed, opened, and all liquid and necrotic material was removed before weighing. The amount of inhibition of tumor growth was then calculated.

One of the methods that can be used to aid in the analysis of test data is the "control chart" procedure, discussed by Merker. Control charts with upper and lower limits set at the 95 per cent level of confidence were constructed for average tumor weight and range in tumor size. When the average tumor weight and range in tumor size for control groups fell within the expected limits of variation, treatment data could be examined with confidence. This procedure was found to be important from the viewpoint of making day-to-day decisions from limited data about the efficiency of potential anticancer agents.

The methods for growing human tumors in the egg for chemotherapy studies were described by Harris. In this procedure human tumors are grown on the chorioallantois of the chick embryo. The procedure involves implantation of a single tumor fragment per egg at 9 days of egg incubation, observation for tumor growth 4 days postimplantation (at which time tumor-bearing eggs are selected for the tests and therapy is initiated), and evaluation, which occurs 6 days after the beginning of therapy. Routinely, a single dose per egg of the test agent is adequate to determine its activity. Multiple injections can be used if a particular situation warrants such a schedule. Combination chemotherapy studies also have been conducted successfully in this system Our previous reports indicated that both HS 1 and HEp 3 responded well to treatment with triethylene melamine.[7] In addition to the testing of the purified and partially purified agents in this system, crude culture filtrates are being screened for antitumor activity. A human bronchiogenic carcinoma and a human adenocarcinoma of the colon recently have been added to this test system. When compared with HS 1 and HEp 3, these tumors represent interesting tumor types with unique responses to certain antitumor agents. When the clinical data are available, comparisons will be made between the response of these tumor types to certain potential antitumor agents and their response in clinical trials using the same agents. Use of the chick embryo as a host to human tumors for chemotherapy studies is unique in that no conditioning agent is required, thus affording greater ease in analysis and interpretation of chemotherapeutic results.

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* Cortone was obtained from Merck Sharp & Dohme, Philadelphia, Pa.

Harris reviewed studies concerned primarily with experimental chemotherapy utilizing hormones and related substances against human tumors growing on the chorioallantois of chick embryos. These preliminary studies have given indications that such tumors may be useful in evaluating potential antitumor agents in these categories. The types of responses encountered in preliminary studies were illustrated by methyltestosterone, cortisone, and progesterone. Since steroids are commonly used as conditioning agents for human tumor growth in other hosts, the chick embryo, which allows human tumor growth without conditioning agents, may provide a unique way of testing steroids and related substances against human tumors outside the patient. Not only is this technique useful as an antitumor test in its own right, but it may also indicate whether the conditioning agents influence the results obtained in conditioned hosts.

Chemotherapy methodology utilizing the Syrian hamster as a host for both human and animal tumors was presented by Handler. Growth and perpetuation of these tumors has also provided a method with which to study the biology of cancer.

During the past year, study of the hamster as a host for experimental chemotherapy of human tumors has been initiated on a small scale by Palm in our laboratory. The purpose of such a study has been twofold: (1) to determine the efficiency of the hamster host-human tumor system for bioassay of potential anticancer agents and (2) to compare the effect in this host of a series of compounds whose effect on the same tumors in the rat already has been determined. This has been considered important in that host-dependent inhibitory activity might be expected to point to ways of studying more specifically the biochemical pathways of tumor inhibition.

The procedure of transplantation used in these studies is as follows. Hamsters weighing 55 to 60 gm. are implanted in the cheek pouch with human tumor, either by trocar (HS 1, HAd, HBR) or by a suspension of homogenized tumor (HEp 3). Cortisone acetate (60 mg./kg.) is administered at the time of implantation and twice more during the following week. Successive transplantations of material through many generations without recourse to alternate hosts are possible. Recently the use of an intramuscular site for maintenance of tumor has been started. Therapy is initiated on the fifth day after implantation and continued daily for 4 to 5 days (HEp 3, HBR) or 6 to 7 days (HS 1, HAd). Evaluation of compound effect is made by comparing the average tumor weight of the treated group with the average tumor weight of the controls. The advantage of post-5-day therapy is that observations of tumor growth in the cheek pouch assure that compounds available in limited amounts need not be wasted on experimental groups in which tumor growth is poor because of contamination or other reasons.

As a host for chemotherapy of the four human tumors mentioned, the hamster has proved thus far to be as efficient as the rat, and presents the advantages of not requiring X radiation as part of the conditioning procedure and of affording good tumor growth in animals over a great age and weight range. Both of these factors make this system more easily amenable to scheduling during the work week and reduces the over-all number of personnel hours per therapy trial.

With respect to inhibitions of these tumors, some differences between results obtained in the rat and hamster have been observed.

Studies concerning the use of the mouse as a host for experimental human tumors were presented by Gallily and discussed in detail by Takayama. This work was in preparation for the possible use of the mouse as a host for tumors during chemotherapy studies. The heterotransplantability of the human epidermoid carcinoma HEp 3 was explored, using different sites of inoculation—subcutaneous, intramuscular, and intraperitoneal. The best results were obtained with intramuscular inoculation. This was achieved when the animals received 100 r total body irradiation and 3.5 to 4.5 mg. of cortisone acetate (Cortone). A comparison of the heterotransplantability of HEp 3 in different strains of mice was made—C57BL/10, C3H/JAX, BALB/c, and the noninbred Swiss mouse. With host conditioning as above, the best results were obtained in strain DBA/2. Strain BALB/c was inferior, although it carried the same histocompatibility allele H-2^d. The transplantability of HEp 3 was studied in Swiss mice under different host treatments made to block the immunological responses. The substances were cortisone, corticosterone, trypan blue, Thorotrast, and X irradiation. Of these, X irradiation plus cortisone, X irradiation plus corticosterone, and Thorotrast plus cortisone appeared to be worthy of further study.

One part of the study reported by Takayama involved the comparison of treated (X irradiation plus cortisone) and nontreated groups of Swiss mice with transplanted human tumor HEp 3. The animals were sacrificed on the first to the seventh days after transplantation. Marked histological evidence of tumor growth was noted on the third day, and gross tumor formation four or five days after transplantation. Comparison was made with the regressive tumor changes, and replacement of tumor cells with fibrous tissue, which occurred in the nontreated group. In a second study, various combinations of mouse-strain and host-conditioning treatment were investigated.

More recently, Merker has begun an exploration of the usefulness of chemotherapeutic tests of intramuscular transplants of HEp 3 growing in the conditioned mouse. These implants grow exceedingly well and attain a weight of approximately 2 gm. in 9 days of growth. Because of the high percentage of tumor "takes" (>95 per cent) and acceptable limits of variation in tumor weight within experimental groups (coefficient of variation <40 per cent), small numbers of tumor-bearing mice have been used for chemotherapeutic trials. From preliminary test data it appears that mouse-grown HEp 3 may be less sensitive than rat-grown HEp 3 to certain select agents.

Finally, Palm discusses the effect of host-conditioning variations on chemotherapeutic results. She points out that the screening of potential anticancer agents against transplantable human tumors in mammals would be virtually nonexistent without procedures for altering the species resistance of animals to heterologous tissues. Three points of importance to experimental therapy have been demonstrated: (1) the growth of one human tumor may be adversely affected by host-conditioning procedures optimum for the growth of a second human tumor; (2) excess as well as undertreatment with cortisone could exert adverse effects on the growth of tumors; and (3) steroids other than cortisone may be less toxic to the host, while allowing tumor growth equal to that resulting from cortisone treatment. Interest also has continued in the ways in which the yeast polysaccharide zymosan affects heterologous tumor growth. The possibility of antispleen sera specifically inactivating immunity-responsible reticuloendothelial cells and thus abrogating resistance to experimental human tumor growth is also under investigation.

Conclusion

It may be said that the human tumor in the heterologous host has become an area for development of methodology and for evaluating compounds, crude filtrates, and agents for anticancer activity. The full usefulness of the heterologous transplantation of human tumors for therapy evaluation cannot be assessed at the present time, however, since this is a new and only partially developed area.

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Part VII. Special Systems

THE UTILITY OF RESISTANT LEUKEMIAS IN SCREENING FOR CHEMOTHERAPEUTIC ACTIVITY*

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The clinical control of even the few types of cancer that do respond to chemotherapy is blocked eventually by the development of resistance. One has only to see such patients as children with acute leukemia brought back temporarily to complete health who then relapse under treatment, to realize how important a problem resistance really is. Elucidation of the biochemical mechanisms whereby this resistance occurs would be of the greatest importance in designing compounds against which the development of resistance would be more difficult, or which would be active against the resistant cell. Perhaps this can be done most effectively by comparing the effect of the agent on the uptake of labeled precursors, or by the incorporation of the labeled agent itself into various chemical components of the resistant and sensitive cells. Although most such studies are possible on human leukemic cells *in vitro*, and some few may be attempted *in vivo*, sensitive and resistant strains of leukemia and tumors in mice offer the most convenient tools for such investigations.

In the past eight years resistance to many chemotherapeutic agents has been developed in various lines of transplanted leukemias and solid tumors in mice by many investigators.[1-8]

In most cases resistance is thought to occur as the result of a random mutation selected out by the presence of the drug, as demonstrated with respect to resistance to A-Methopterin in leukemia L 1210 by Law.[9] From his studies two generalizations are worth mentioning: (1) that several variants stemming from the same parent line of leukemia may develop resistance to the same agent by different biochemical mechanisms, and (2) that there may be great differences in the ease with which resistance to a given agent may be induced in various lines of leukemia. A case in point would be resistance to the glutamine antagonist 6-diazo-5-oxonorleucine (DON), which Potter and Law[2] have shown develops in a single step in leukemias P 815 and 70429, but which we have not been able to develop in leukemia L 1210 after 36 generations (TABLE 1).

The need at the present time in the clinical chemotherapy of leukemia is for new compounds that will be active against leukemias that are initially resistant or have become resistant to conventional antimetabolite chemotherapy, particularly the folic acid, purine, and glutamine antagonists. Since, with one or two possible exceptions,[10] these antimetabolites have been a dismal failure in the chemotherapy of patients with metastatic carcinomas and other

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TABLE 1
INABILITY OF LEUKEMIA L 1210 TO DEVELOP RESISTANCE TO DON*

Generation	Treatment (mg./kg.)	Mean survival time, days
0	Control	13.3
	DON 1	21.4
	A-Methopterin 3	26.1
36†	Control	8.4
	DON 1	19.3
	A-Methopterin 3	15.3
	6-MP 12.5 and DON 0.25	14.1

* All doses were given I.P. 3 times weekly.
† Generations through mice treated with DON 1 mg./kg. 3 times weekly.

solid tumors, it is obvious that here, too, we need agents capable of exerting effects on cells that are relatively insensitive to these conventional antimetabolites. For this reason it would seem advantageous to include in the primary screening of antitumor agents either a leukemia or a solid tumor in which resistance has been induced to the conventional antimetabolites, such as A-Methopterin and 6-mercaptopurine (6-MP). A positive effect on such a line of leukemia would demonstrate not only that a new compound or antibiotic filtrate had an antileukemic or antitumor activity, but also that this effect was exerted by a mechanism different from those of the purine antagonists or the folic acid antagonists. In addition, as was first demonstrated by Law,[11] such a line of resistant leukemia might have increased collateral sensitivity, which would allow it to pick up antileukemic agents that might be without effect against the original line sensitive to A-Methopterin and 6-MP. Although studies using a doubly resistant line (FIGURE 1), that is, one resistant to both A-Methopterin and 6-MP, are just beginning in our laboratory and we have not as yet found a compound that is both specifically active against the doubly resistant strain and inactive against the parent sensitive strain, it is conceivable that such a compound may be picked up by this variant.

The second use of resistant strains, this time as a secondary screening procedure, is to demonstrate cross resistance between a new compound or filtrate and the known conventional agents. From the point of view of the clinician this is extremely important since, if a new compound were discovered with cross resistance to the known antifolics, we should be far less interested in it than we should be if it were acting by another mechanism. The excellent carry-over between mouse and human leukemia has been demonstrated in this respect; spontaneous leukemias in children or strains of transplanted leukemia in mice that have developed resistance to A-Methopterin show cross resistance to Aminopterin, amino anfol, adenopterin, 2,4-diamino-5(3′,4′-dichlorophenyl)-6-methyl pyrimidine, and all the other folic acid antagonists studied thus far. A similar situation exists with the purine antagonists; in both patients and mice, leukemias resistant to 6-MP are cross-resistant to thioguanine, and chloropurine. On the other hand, the fact that the fluorinated pyrimidines[12] are active in mice against variants of leukemia L 1210 made resistant to both the purine antagonists and the folic acid antagonists

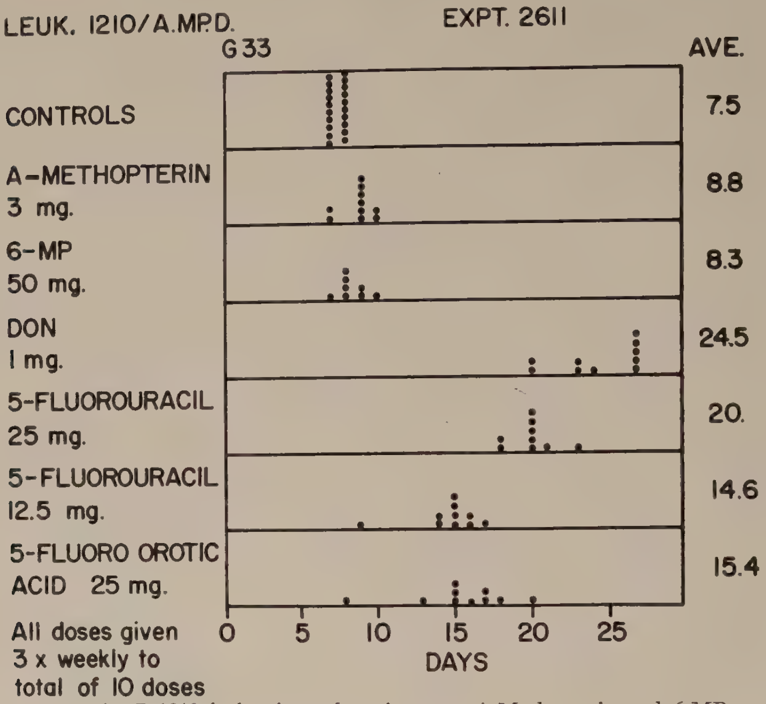

FIGURE 1. L 1210 leukemia made resistant to A-Methopterin and 6-MP.

(FIGURES 2 and 3) makes them extremely interesting to the clinician and leads to the hope that they will be useful in patients with acute leukemia who have become resistant to conventional agents.

A third sphere of usefulness of the resistant strains of leukemia is in the determination of collateral sensitivity and slight degrees of impaired sensitivity as indications of the sequence in which these agents should be employed. Thus Law's studies,[11] which indicated increased collateral sensitivity to A-Methopterin in strains of leukemia L 1210 made resistant to or dependent on 6-MP and the lack of collateral sensitivity to 6-MP in strains of leukemia made resistant to A-Methopterin, would suggest that the clinical sequence should be, first, 6-MP, followed by A-Methopterin, rather than vice versa. Studies in children are as yet inadequate for determining the preferred sequence of treatment. However, from the work of Ellison[13] in adults with acute leukemia, it appears that among those patients whose disease has first responded to 6-MP and then developed resistance there is a considerably higher percentage of remissions—on the order of 30 per cent—to A-Methopterin than would have been expected among patients previously untreated. FIGURE 1 demonstrates the markedly increased collateral sensitivity to A-Methopterin and the excellent responses also to DON and fluorouracil in a line of L 1210 made resistant to 6-MP.

A fourth use of resistant lines of leukemia is in determining roughly the

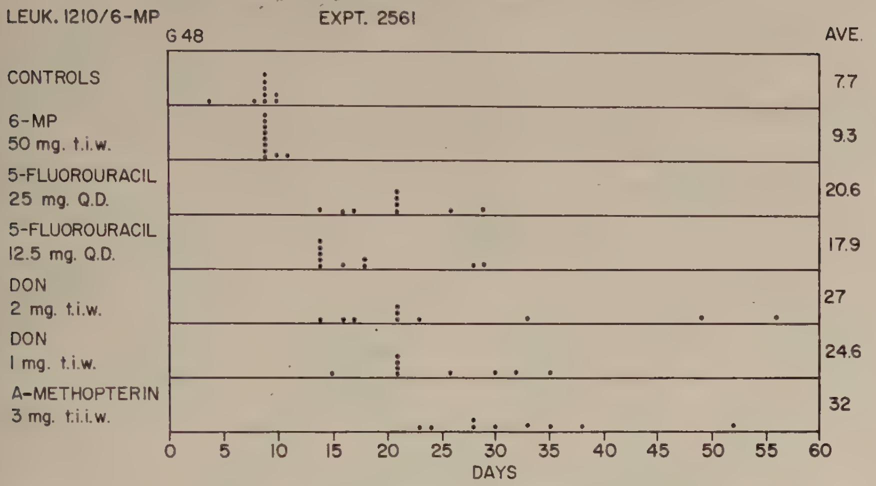

FIGURE 2. L 1210 leukemia made resistant to 6-MP.

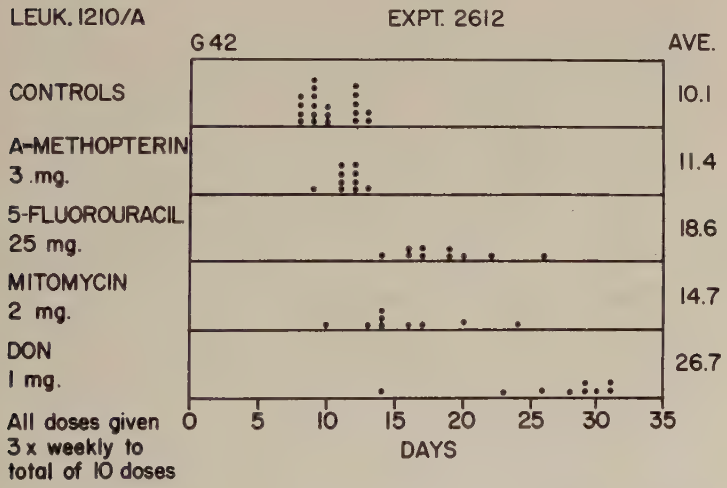

FIGURE 3. L 1210 leukemia made resistant to A-Methopterin.

mechanism of action of new chemotherapeutic agents. For instance, biochemical studies by Heidelberger *et al.*[14] have shown that the fluorinated pyrimidines act to prevent the conversion of a uracil-containing compound to a thymine-containing compound. The folic acid antagonist A-Methopterin also acts in pyrimidine synthesis in roughly this same general area. The fact that the fluorinated pyrimidines are still active against A-Methopterin-resistant strains of leukemia (FIGURE 1 and 3) would suggest, however, that the exact loci of antileukemic action are different for the two classes of compounds.

The mechanism of the antileukemic action of microbiological filtrates can be studied by techniques for microorganisms similar to those mentioned else-

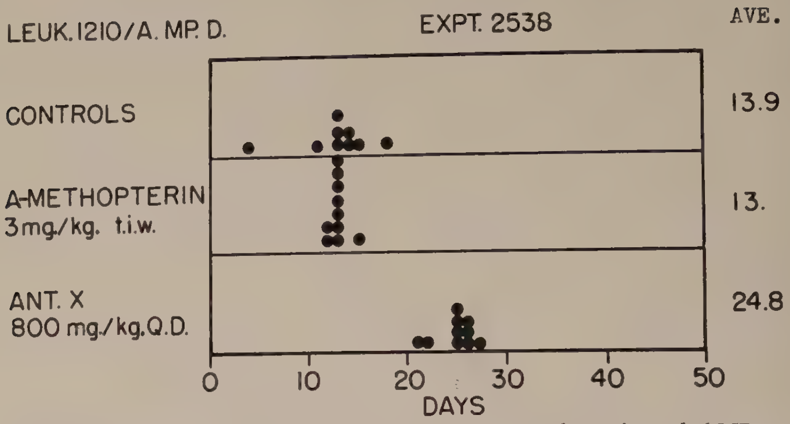

FIGURE 4. L 1210 leukemia made resistant to A-Methopterin and 6-MP.

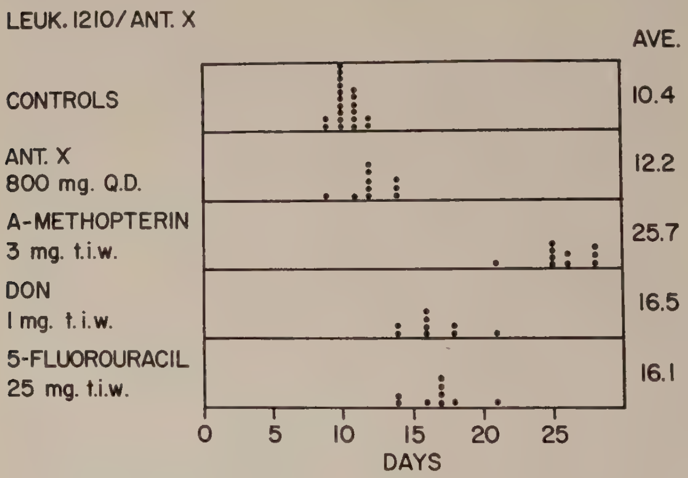

FIGURE 5. L 1210 leukemia made resistant to antibiotic X.

where in this monograph by F. M. Schabel. Thus, by testing an active unidentified antibiotic preparation against strains of leukemia made resistant to known chemotherapeutic agents (FIGURE 4) and by testing these chemotherapeutic agents against a strain of leukemia made resistant to the new filtrate (FIGURE 5), the unidentified antibiotic was demonstrated to be exerting its antileukemic effect, not by virtue of an antifolic, antipurine, antipyrimidine, or antiglutamine action, but by some other mechanism.

A fifth potential use of the resistant strains of leukemia would be in studying the ability of a new agent to reverse resistance previously acquired to another drug. It is conceivable that treatment of a line of resistant leukemia with a new agent for a few generations may bring about a return of sensitivity to the original agent.

Summary

Lines of leukemia made resistant to the conventional antileukemic agents are useful in the following ways: (1) to detect, by the mechanism of collateral sensitivity, antileukemic activity not demonstrable by the parent line; (2) to demonstrate cross resistance to conventional agents; (3) to detect collateral sensitivity or lack of it as an aid in determining the sequence of the clinical use of the drug; (4) to study the mechanism of action of a new agent; and (5) to study the possible loss of resistance to one agent while developing resistance to a second.

Finally, we emphasize again that perhaps the most promising use of resistant lines of leukemias and tumors lies in the biochemical comparison of these with the sensitive parent lines in an attempt to elucidate the mechanisms of resistance. As mentioned previously, there may well be several mechanisms for the development of resistance to a given agent, but a knowledge of these mechanisms might allow the synthesis of an antileukemic agent that could surmount these barriers to successful control. Certainly, in the chemotherapy of cancer, studies of this type should be given a high priority.

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Discussion of the Paper

Murray J. Shear (*National Cancer Institute, Public Health Service, Bethesda, Md.*): Resistance is important and warrants more careful attention. One might think there were at least three kinds of resistance. In the first case, an agent such as nitrogen mustard is effective against Hodgkin's disease, but not against carcinoma or leukemia. In the second case, an antifolic is effective against acute leukemia in children but proves, after study of perhaps 300 cases, that it causes dramatic improvement in about 30 per cent of the cases, partial improvement in another 30 per cent, and shows resistance in still another 30 per cent. In the third type of resistance, a given type of tumor in a human or mouse responds very dramatically to initial treatment but later, after recurrence in the same disease in the same individual, no longer responds.

Perhaps throughout these pages we have been a little too vague in our considerations of cancer, as if it were a single disease. Not only are there many forms of neoplastic disease that respond differently to different chemotherapeutic agents, but responses are different even in the same kind of neoplastic diseases morphologically and clinically. Also, in the same individual the response may be different at different times. As Lettré remarked earlier in these pages, the findings are valid only for the one tumor on which the compound has been tested.

Lloyd W. Law (*National Cancer Institute, Public Health Service, Bethesda, Md.*): To enlarge somewhat on the problem Burchenal has presented, I point out that the problem is not simple and that we may be making unwarranted generalizations from single neoplasms or observations obtained from a single neoplasm. The phenomenon of acquired resistance, as described by Burchenal, has been generally observed in a fairly wide spectrum of neoplasms and against a wide spectrum of agents, for example, antifolic, antipurine, antiglutamine antagonists, pyrimidine antagonists, TEM, and several nitrogen mustard derivatives. In a given morphologic form of neoplasm, for instance, the lymphocytic neoplasm in the mouse, one finds those that respond dramatically to a given drug and others that are completely resistant to it. As a matter of fact, we have seen some transplantable neoplasms that appear to be naturally dependent; that is, at least they grow optimally in the presence of 8-azaguanine, for example, without ever being exposed to the drug. Perhaps this problem should receive more attention. Is it possible, for example, to increase the sensitivity of neoplasms to drugs?

I think we should pay more attention to the origin of resistant populations of neoplasms. It is now clear that the variables concerned with the development of resistance are those confined to the cells and not the host; for instance, the development of resistance in mouse neoplasms in tissue culture holds, or it is carried over to *in vivo* studies in the mouse.

In reality, little has been done, as far as patterns of resistance are concerned, but these are necessary if one wishes to arrive at some rationale for the treatment of neoplasms. We have reported several different patterns of resistance that others also have observed. One of these is the development in discrete stepwise increments of resistance in certain neoplasms to certain drugs, which resembles penicillin resistance in bacteria. Another is the rapid development of resistance to high levels of a drug, especially to the glutamine antagonists azaserine and DON. Yet another, which is observed among bacteria, is a resistance to compounds, which is rather unstable and reverts, usually slowly, in the absence of the drug. Most patterns, however, have been heritable and stable in the absence of the drug, which probably indicates the operation of genetic mechanisms.

One would like to know, for example, regarding childhood leukemia, whether the resistance that appears is really heritable and stable, or is a rapid population change that may in time revert to sensitivity. I am not sure that this is known.

There are apparently two alternatives for the origins of resistance. One would be considered genetic, since in it the drug really acts only as a selective mechanism. The models used in bacterial studies certainly indicate the importance of this mechanism. The recombination studies and the replica plating of Lederberg and the transformation studies of Hotchkiss show that there is a genetic or a genic basis for resistance, at least in bacteria.

There has been little attempt to define the mechanisms of resistance for neoplasms, of course, because of the difficulty of dealing with somatic cells. We have used one test—the fluctuation test of Luria and Delbrück, and only one neoplasm—and arrived at the conclusion that resistance here probably resulted from mutation and selection; but this is for the given neoplasm only, and I think we should not generalize from it. My associates and I have shown in our laboratory, for example, that by using a tetraploid neoplasm, the lymphocytic neoplasm P 288, resistance is developed easily; this has been shown also with the Ehrlich neoplasm, which has a considerable amount of ploidy. Consequently, the genes involved in the development of resistance in these neoplasms are probably dominant genes.

Knowing the origin or determining the origin, whether genetic on the one hand, or physiological -for example, the result of adaptive enzymes—on the other, will be feasible, I think, in the near future with the development of tissue culture techniques and the development of specific markers on the tissue culture cells, which at least will allow one to determine transformation or transduction mechanisms.

All this knowledge seems to me necessary before we arrive at some rationale in the treatment of neoplasms in an attempt to circumvent or to preclude the phenomenon of resistance.

Burchenal has mentioned some possibilities of exploiting the character of resistance. One is the use of collateral sensitivity studies whereby one determines whether the development of resistance to a drug will produce predictable changes in metabolic pathways leading to increased sensitivities to other drugs. One example that Burchenal mentioned is the increased sensitivity to A-Methopterin of antipurine-developed resistance, and another is the increased sensitivity to azaserine of a cortisone-resistant mouse neoplasm. These are single neoplasms, and I think it would be unwise to generalize from single neoplasms. We need more information on a variety of different forms of neoplasms.

The use of antimutagenic agents in combination with carcinostatic drugs should be kept in mind. For example, Novick and Szylard have shown that guanosine will supress the mutagenic effects of caffein and other materials. If one thinks of these in terms of genetic changes, it may be valuable to attempt to use antimutagenic substances.

Finally, the use of combinations of drugs, for instance, of two drugs in combination when neither drug alone shows an inhibitory effect in a neoplasm, has recently been observed to produce striking effects, and this approach merits further investigation.

COMMENT: Regarding the problem of resistance: the difficulty lies in the possibility of selecting cells from a mixed population. Some of the studies presented by Hutchison indicated very clearly this variability among several cell lines that are selected for resistance. Recent studies by Hauschka at Roswell Park Memorial Institute, Buffalo, N. Y., indicate considerable variation in examining the cytology of these cells. Hauschka has observed each combination of variation in which a resistant population may have the same number of chromosomes, fewer chromosomes, or more chromosomes than the parent population. Perhaps this detailed examination of the cells should be one of the important controls. In addition, it becomes more important to use clonal selection before beginning the selection for resistance, which gives better control in this type of experiment.

SHEAR: One of the fascinating challenges in cancer is a consistant distinction from most normal tissues. When malignant cells divide, the daughter cells do not have the same size, shape, nuclei, or staining properties. Therefore, if one finds the chemical agents that can kill 90 per cent of the tumor cells, perhaps some of the daughter cells may be sufficiently different from the parent cells, and will not be susceptible to these agents.

FRANK M. SCHABEL, JR. (*Southern Research Institute, Birmingham, Ala.*): The problem of drug resistance is of great practical importance in human leukemias. Such resistance in experimental mouse leukemias, especially Leukemia 1210, has been demonstrated repeatedly and studied extensively. A 6-mercaptopurine-resistant line of Adenocarcinoma 755 developed in our laboratories by J. R. Thomson is relevant to the problem.

For a number of years my associates and I have been interested in purine antagonists as anticancer agents, especially in the effects of purine antagonists on Adenocarcinoma 755, which is particularly sensitive to purine antagonists.

Since 6-mercaptopurine (6-MP) has the highest chemotherapeutic index, that is, the ratio of greatest nontoxic dose to the smallest tumor-inhibiting dose, of any experimentally effective anticancer agent, we were interested in whether or not we could develop a 6-mercaptopurine-resistant line of Adenocarcinoma 755 (Ca 755).

Serial transplants of this tumor maintained under continuous treatment with 6-MP developed resistance to the drug; we have designated this tumor 755/6-MP. TABLE 1 shows the results of experiments in which the drug-

TABLE 1

INHIBITORY EFFECTS OF SELECTED COMPOUNDS ON ADENOCARCINOMA 755 AND 755/6-MP

Compound	Dosage (mg./kg.)	755		755/6-MP	
		Avg. tumor wt. (mg.) T/C	Tumor wt. % of control	Avg. tumor wt. (mg.) T/C	Tumor wt. % of control
6-Mercaptopurine	20.0	34/1005	3	874/1583	55
6-Mercaptopurine riboside	17.0	9/466	2	589/1227	48
6-Thioguanine	1.0	58/1034	6	770/1370	56
8-Azaguanine	75.0	103/1080	10	72/1300	6
4-Aminopyrazolo-(3,4-*d*)pyrimidine	20.0	13/1053	1	48/923	5
A-Methopterin	1.5	929/946	98	576/968	59
Azaserine	8.0	398/860	46	890/1094	82
DON	0.075	476/920	52	1253/1734	72

sensitive and drug-resistant tumors were subcutaneously implanted bilaterally in the same host and treated with several anticancer agents.

It can be seen that, although this tumor is not completely resistant to 6-MP, it does have a considerable degree of resistance. Inhibitions to less than 10 per cent of the control are seen consistently in the parent drug-sensitive tumor treated with as little as 1 to 2 mg./kg./day with 6-MP. It is also resistant to 6-MP riboside, high levels of the 6-MP riboside inhibiting both, but lower levels markedly inhibiting only the drug-sensitive line. The resistant tumor is likewise cross-resistant to 6-thioguanine, 6-methylmercaptopurine and its riboside, 6-chloropurine and its riboside, 6-benzylmercaptopurine, 9-ethyl-6-mercaptopurine, and 6-ethylmercaptopurine. Unlike the 6-MP-resistant line of Leukemia 1210, which Law reported was cross-resistant to 8-azaguanine,[1] the 755/6-MP is not cross-resistant to 8-azaguanine. Neither is it cross-resistant to 4-aminopyrazolo-(3,4-*d*)pyrimidine. Unlike the 6-MP-resistant and 8-azaguanine-resistant lines of Leukemia 1210, which are more sensitive to the action of A-Methopterin than the parent drug-sensitive L 1210,[1] the 755/6-MP is not sensitive to A-Methopterin. Perhaps this is not surprising, since neither is the parent drug-sensitive line of Ca 755. Neither the parent drug-sensitive Ca 755 or 755/6-MP is sensitive to azaserine or 6-diazo-5-oxo-L-norleucine (DON).

We had hoped that studies with this 6-MP-resistant line of Ca 755 would reveal compounds with selective toxicity for the drug-resistant tumor, but none has yet been found. We feel that this 755/6-MP tumor is a useful tool, however, since it allows simultaneous testing of the drug-sensitive and drug-resistant tumor in the same host. It is to be hoped that by using these two tumors in the same host it will be possible to demonstrate selective toxicity of some anticancer agents for the drug-resistant line.

Observed cross-resistance or lack of cross-resistance of the 755/6-MP tumor allows some preliminary deductions concerning the possible mode of action of new purine antagonists.

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Dorris J. Hutchison (*Sloan-Kettering Institute for Cancer Research, and Sloan-Kettering Division, Cornell University Medical College, New York, N. Y.*): Numerous suggestions for the use of antimetabolite-resistant mouse neoplasms have been presented and discussed by Burchenal, Schabel, and Law. Indeed, it would be ideal to have a biological system that could answer Burchenal's proposals. Our work with antimetabolite-resistant strains of *Streptococcus faecalis* suggests that a single resistant culture or system cannot be used, for there appear to be many metabolic alterations responsible for the ultimate, unfortunate phenomenon of resistance to a specific antimetabolite.

For some time we have been pursuing the problems of the delineation of the alterations that occur in antimetabolite-resistant lines of the L 1210 mouse leukemia. Results of some of these studies are tabulated in table 1. Three lines selected on the basis of A-Methopterin resistance, that is, selected by passage of the L 1210 leukemia through Bittner's D2BC mice treated with A-Methopterin, are also resistant to 6-MP. Line IV is less sensitive to 5-fluorouracil, but lines VII and IX have retained some sensitivity to this uracil analogue.

Three lines selected in the presence of 6-MP are also noteworthy. Lines III and VIII show a slight increase in sensitivity to A-Methopterin and are similar to L 1210 (V) in their response to 5-fluorouracil, while line X is resistant to all 3 antimetabolites. The 2 double mutants, that is, those selected under the stress of both 6-MP and A-Methopterin, are very similar in their response to the 3 drugs used in this experiment.

Even though azaserine produces a very slight effect against the L 1210 (V) leukemia, a resistant line was selected. This line XII is most interesting in that it is resistant to A-Methopterin and 6-MP and has a slightly increased sensitivity to 5-fluorouracil.

All of these leukemias, with the exception of the three 6-MP-resistant lines,

Table 1
Characterization of Resistant Lines of L 1210 Mouse Leukemia

Line	Average survival time (AST) in days (25 mice in each group)						
	Control	A-Methopterin 3 mg./kg.		6-MP 40 mg./kg.		Fluorouracil 25 mg./kg.	
	AST	AST	Per cent change	AST	Per cent change	AST	Per cent change
L 1210 (V)	10.2	23.1	+126	16.9	+66	18.1	+77
L 1210/A (IV)	14.0	13.4	−4	13.1	−6	18.3	+31
L 1210/A (VII)	14.4	13.1	−9	13.6	−6	23.3	+62
L 1210/A (IX)	12.3	12.5	+2	11.9	−3	18.0	+46
L 1210/MP (III)	10.8	27.6	+156	11.1	+3	17.7	+64
L 1210/MP (VIII)	10.7	27.2	+154	10.5	−2	17.8	+66
L 1210/MP (X)	11.8	14.3	+21	11.4	−3	14.7	+25
L 1210/MP/A (VI)	13.4	12.4	−8	11.9	−11	20.0	+49
L 1210/MP/A (XI)	14.3	16.2	+13	12.7	−11	24.3	+70
L 1210/Azas. (XII)	13.6	14.1	+4	13.6	0	25.0	+84

appear to be less virulent than the parent line, as evidenced by an increased average survival time in the control mice. The results observed in the D2BC mice have also been confirmed in DBA/2 mice.

At the present time, studies on further characterizations are proceeding on these ten lines of the L 1210 mouse leukemia. Since the results to date suggest that the lines are quantitatively different, it would be advantageous to discover some qualitative differences. Once these investigations are completed, it should be possible to select five or more resistant lines that have several different alterations, which could then be used as secondary screens for evaluating new anticancer agents and also serve as aids in the study of the mode of action of the potential chemotherapeutic substances.

SHEAR: The trouble with this general problem is that it bristles with too much resistance of too many different types. If, however, we are going to overcome them, it is necessary that we learn the nature of their resistance.

Whether one reacts to the work being presented here with a feeling of disappointment or encouragement depends upon what one expects. Some people not only expect that screening procedures will identify drugs of clinical usefulness, but that they also will reveal information about the drug's usefulness for such cases as bone sarcoma in females between 20 and 30, or for glioma in males between 10 and 20. There is no ideal screening procedure for pinpointing the clinical usefulness of drugs, and all different procedures are open to criticism. For those of us who approach the program with modest expectations, the realization that more and more investigators are grappling with the problems involved, and that each one in his own way is making some headway is a source of great encouragement.

CHARACTERIZATION OF THE SPECIFICITY OF ACTION OF TUMOR-INHIBITING COMPOUNDS

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Introduction

The present situation in experimental and clinical cancer chemotherapy confronts us with the problem of finding methods by which to determine the intrinsic value of the results obtained experimentally with a spectrum of animal tumors that is still regarded as affording the most appropriate approach to the problem.[1] In other words, the inhibition of transplantable or spontaneous animal tumors, even by compounds with a relatively large therapeutic range, is probably insufficient evidence of specificity, as long as normal tissues of the animal are damaged at the same time.

Considerable work has been devoted to tracing the enzymatic pattern[2] and to studying the nature of nucleic acid synthesis.[3-11] Although it is known that major biochemical differences exist between tumor and normal tissue, for example, the differing behavior of respiration and glycolysis[12] or the differing calcium content,[13, 14] it has not yet been possible to exploit these differences for chemotherapeutic purposes. Only in the case of certain hormone-dependent tumors has a form of specific therapy been tentatively outlined.

For this reason, any method in which empirical results offer new prospects of a rational approach seems worth considering. The present paper discusses one possible method of comparative investigation in male rats using the Walker carcinosarcoma 256, the uterine epithelioma T-8 Guérin, the Flexner-Jobling carcinoma for malignant growth, and the foreign-body granuloma for nonmalignant, rapidly growing tissue. Obviously, in such comparative studies any normal embryonic, adult, or regenerating tissue can be used, either *in vitro* or *in vivo*, as a counterpart to tumors. One of the essential advantages of the experimental setup discussed below is that it seems to make it possible to evaluate comparatively, quantitatively, and qualitatively the different results obtained against malignant and nonmalignant tissues in identical animal species, using identical routes of administration.

Methods

The methods used have been described previously.[15-17] The thirty compounds studied are listed in TABLE 1 according to Burchenal's classification.[18]

The substances were administered daily, either in solution or in suspension in saline by the intraperitoneal and oral routes, for 6 days in doses increasing to the maximum tolerated, that is, to the highest dose at which at least 51 per cent of the tumor-bearing animals survived the complete duration of the treatment or showed tumor inhibition of more than 75 per cent in comparison with the controls. Treatment of the granuloma-bearing animals was carried out over the same dose range. The maximum dose used was 300 mg./kg./day,

TABLE 1*

1. Antimetabolites
   - (a) Compounds presumably affecting *de novo* synthesis of purines and pyrimidines
     - Folic acid antagonists
       - 4-Amino compounds
       - Aminopterin
     - Glutamine antagonists
       - O-Diazoacetyl-L-serine (azaserine)
       - 6-Diazo-5-oxo-L-norleucine (DON)
   - (b) Compounds presumably affecting incorporation of purines and pyrimidines into nucleic acid
     - Purine antagonists
       - 2,6-Diaminopurine
       - 8-Azaguanine
       - 6-Mercaptopurine (6-MP)
       - 1-Methyl-4-aminopyrazolo-(3,4-*d*)pyrimidine
       - Adenine
     - Pyrimidine antagonists
       - 2-Thiouracil
       - 5-Fluorouracil
   - (c) Compounds presumably acting as amino acid antagonists
     - DL-Ethionine
   - (d) Compounds possibly acting as antimetabolites
     - Urethan
     - Actinomycin C
     - Actinomycin D
     - Actinomycin J
     - Cinerubin A
2. Polyfunctional alkylating agents
   - (a) Halogenated alkylamines
     - Di-(2-chloroethyl)methylamine·HCl
   - (b) Halogenated arylamines
     - ω-[*N*,*N*-Di-(2-chloroethyl)-*p*-aminophenyl]butyric acid (CB 1348)
     - *N*,*N*-Di-(2-chloroethyl)-*p*-aminophenylalanine (CB 3025)
   - (c) Halogenated alkylamine oxides
     - Di-(2-chloroethyl)methylamine oxide·HCl (Nitromin)
   - (d) Triethylenemelamine (TEM)
   - (e) Triethylenephosphoramide (TEPA)
     - Ethyleniminoquinones
       - 2,5-Bis-ethyleniminobenzoquinone
       - 2,5-Bis-ethyleniminohydroquinon
       - 2,5-Bis-acetamino-3,6-bis-ethyleniminobenzoquinone
       - 2,5-Bis-propoxy-3,6-bis-ethyleniminobenzoquinone (E 39)
   - (f) 1,4-Dimethanesulphonoxybutane (Myleran)
3. General cell poisons
   - Colchicine
   - Demecolcine
4. Hormones
   - Adrenal hormones
     - Cortisone

* Azaserine and DON were supplied by H. W. Bond, Bethesda, Md.; 5-fluorouracil, by A. Studer, Basle, Switzerland; actinomycin D, by B. H. Waksman, New Brunswick, N. J.; actinomycin J, by T. Kodama, Tokyo, Japan; CB 1348, CB 3025, and Myleran, by A. Haddow, London, England; and Nitromin, by N. Brock, Brackwede, Germany. The remaining compounds were either made in our own research laboratories or purchased.

even in cases in which the maximum tolerated dose had not been reached. If allowance is made for these factors, it is possible to compare quantitatively and qualitatively the dose/effect curves. The standard for minimum inhibition of tumor growth was arbitrarily fixed, that is, when, for at least 1 of

TABLE 2

Type	Growth inhibition of	
	Granuloma	Tumor
1	∅	∅
2	+	∅
3	+	+
4	∅	+

Symbols: + = growth inhibition; ∅ = no growth inhibition or stimulation.

the 3 tumors used, an index of 0.6 or less was reached following intraperitoneal and oral administration. An index of 1 indicates no effect; an index of 0 indicates total inhibition. The differences in the absolute values of the tumor diameters must be significant at a value of p* < 0.001. Minimum inhibition of granuloma growth was considered to have occurred when the granuloma of treated animals weighed at least 60 mg. less than the controls showing a maximum possible inhibition of 200 mg. Here, too, the difference must be significant at a value of $p < 0.001$.

In order to establish the therapeutic range of any given compound after intraperitoneal and oral administration, the cumulative toxicity following one daily dose for six consecutive days was determined at the end of the second day after the last dose—as in the tumor experiments—on normal male animals that were in all respects comparable to the tumor- or granuloma-bearing rats.

Results

With this experimental setup it is possible to distinguish qualitatively among four possible types of action (TABLE 2). The following figures illustrate the results obtained.

Examples of substances of Type 1, that is, substances not producing any significant growth inhibition either of the tumors or of the granuloma, are urethan (FIGURE 1) and 2-thiouracil (FIGURE 2).

Cortisone is an example of a substance of Type 2 (FIGURE 3), which inhibits only the growth of the foreign-body granuloma, without causing significant tumor inhibition. The compound 1-methyl-4-aminopyrazolo-(3,4-*d*)pyrimidine (FIGURE 4) conforms to this type only partially, that is, only by one of the two alternative routes of administration. In response to oral administration very pronounced granuloma inhibition occurs, whereas no effect is exerted on tumor growth. After intraperitoneal administration, however, both types of growth are inhibited.

Type 3, which is characterized by concomitant inhibition of tumor and granuloma growth in the case of both routes of administration, is exemplified by actinomycin C (FIGURE 5). Characteristic of this substance are the rather steep decline in the dose/effect curve and the narrow therapeutic range. Another example of Type 3 is 2,5-bis-acetamino-3,6-bis-ethyleniminobenzo-

* Symbol: p = probability.

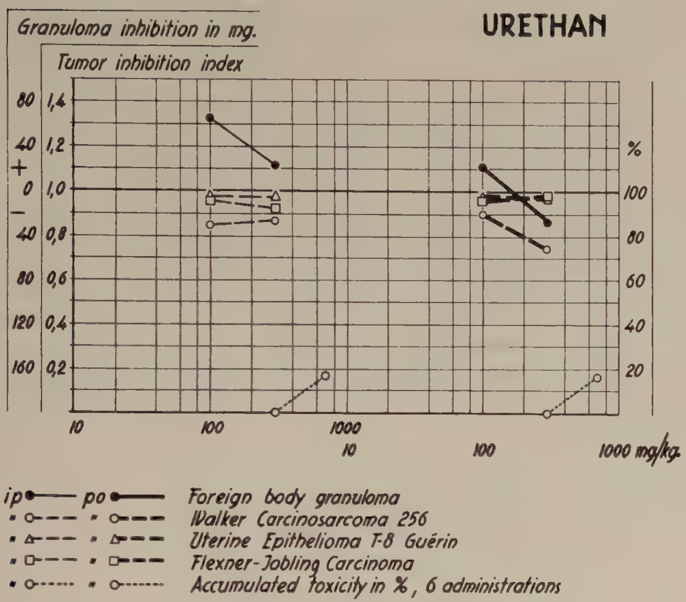

FIGURE 1

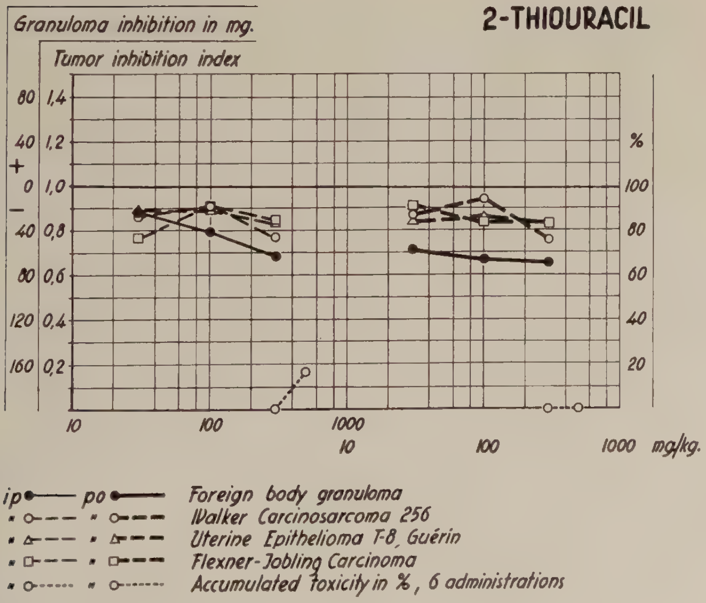

FIGURE 2

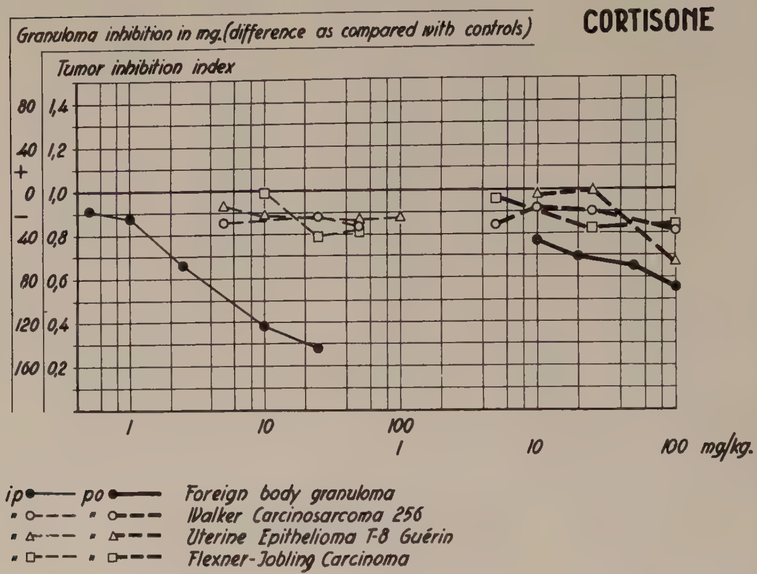

FIGURE 3

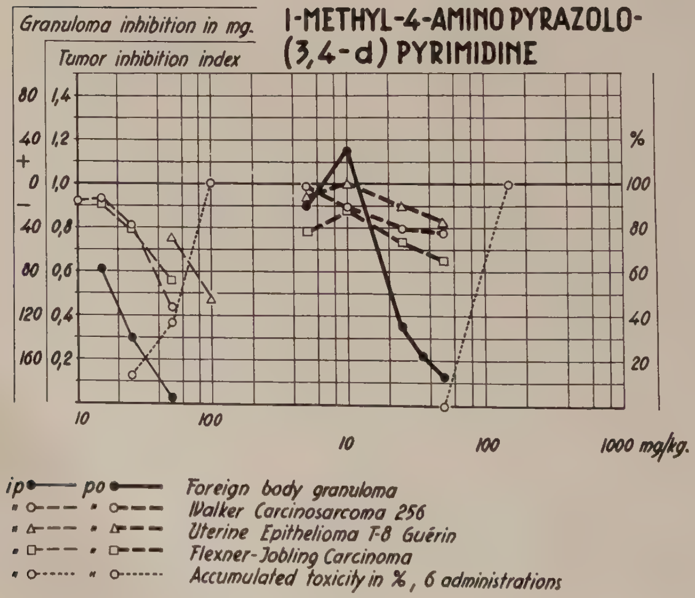

FIGURE 4

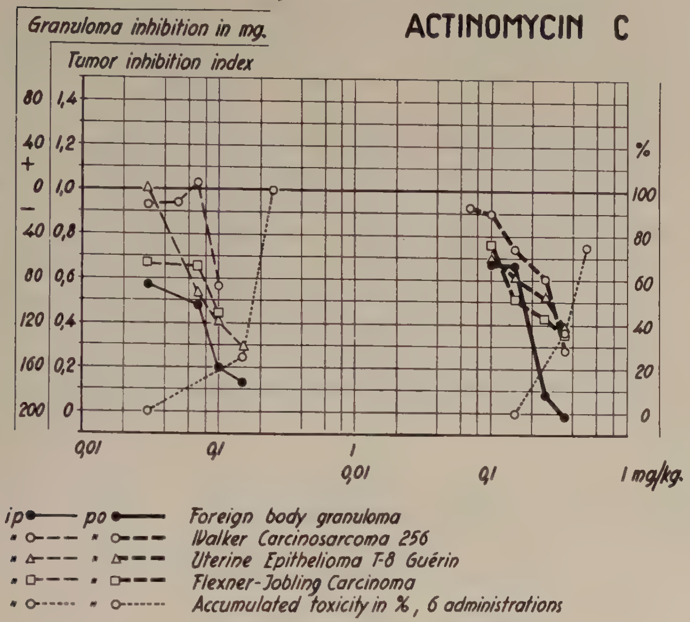

FIGURE 5

quinone (FIGURE 6). In general, the slope of the dose/effect curves for this compound is qualitatively identical with that of actinomycin C. However, several quantitative differences appear.

(1) The dose/effect curves, particularly after oral administration, are flatter.

(2) A larger therapeutic range is evident, especially after intraperitoneal dministration.

(3) The ratio between the inhibition of the Walker carcinosarcoma 256 and the granuloma is more favorable, especially following intraperitoneal aplication when measured at ED_{50}, or at a dose of 30 mg./kg., for example.

(4) It is also apparent that, after oral administration of this compound, ats bearing different tumors show a different tolerance level to cumulative oxicity; the maximum tolerated doses for rats with uterine epithelioma T-8 uérin are 30 mg./kg., with the Walker carcinosarcoma 256, 70 mg./kg., nd with the Flexner-Jobling carcinoma 100 mg./kg.

No substances showing complete conformity to Type 4 have yet been found. artial conformity to type has been observed, however, in some cases after ither intraperitoneal or oral administration, one example being cinerubin A FIGURE 7). The diagram of cinerubin A is comparable to that of actinomycin C ut, after oral administration, no inhibition of the granuloma is observed, lthough the trend of the dose/effect curve for the granuloma is the same as t is for the tumors.

Aminopterin (FIGURE 8) shows a more pronounced difference with respect

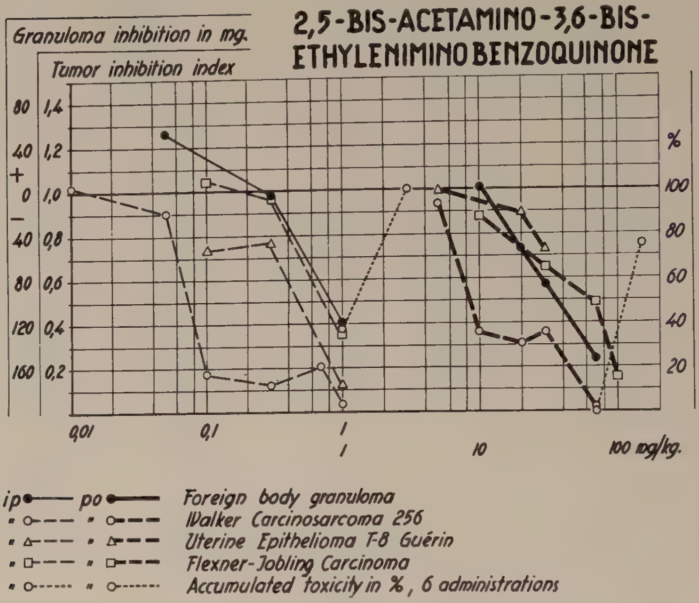

FIGURE 6

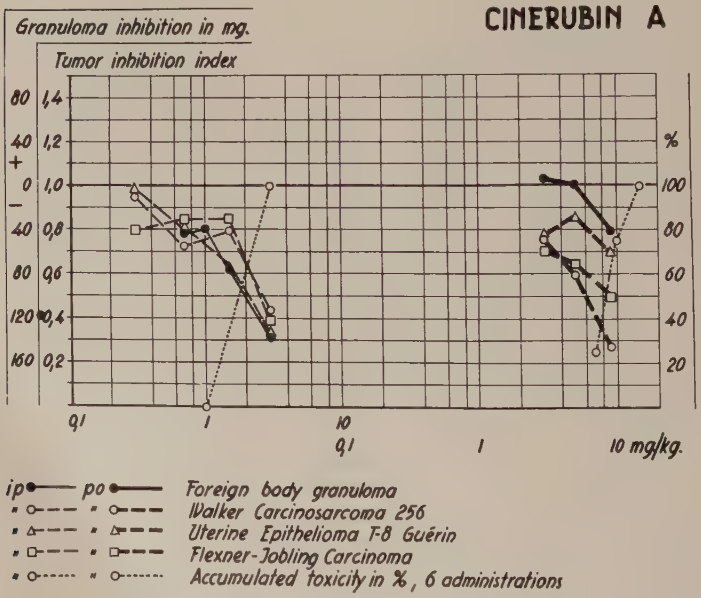

FIGURE 7

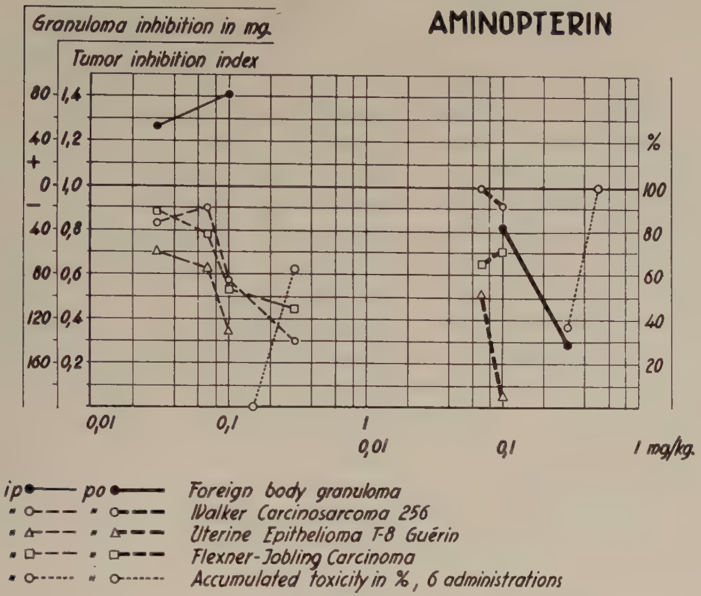

FIGURE 8

to the absence of granuloma-growth inhibition following intraperitoneal administration. The significance, if any, of only partial conformity to type is discussed later.

Discussion and Conclusions

The results obtained are tabulated in TABLES 3 and 4. The activity displayed by nine compounds (TABLE 3) belonging to disparate chemical groups lid not conform completely to type. Like the examples shown in FIGURES 4, 7, and 8, other substances, as shown in TABLE 3, exhibit two types of action lepending on the routes of administration. Aminopterin, cinerubin A, CB 1348, CB 3025, and TEPA show a partially specific tumor-inhibiting activity, Type 4, and, consequently, it might be profitable to carry out further studies n these chemical groups in the hope of attaining specificity of action. The esults at present, however, are only of limited value, since they are most robably due to differences in the manner in which the substances are absorbed n the organism as one example among many other possibilities.

Of the thirty substances that were investigated systematically, more than ne half show Type 3 action, that is, they inhibit tumor growth, but with oncomitant inhibition of granuloma growth. In other words, they lack pecific tumor-inhibiting activity.

Results similar to those we obtained with 2,5-bis-acetamino-3,6-bis-ethylniminobenzoquinone, which shows different cumulative toxicity values lepending on the type of tumor implanted in the rats, have recently been lescribed by Riley,[19] who thinks that ". . . the difference in survival time

TABLE 3*

Type 1	Type 2	Type 3	Type 4
		P.O. Aminopterin	I.P. Aminopterin
	I.P. 2,6-Diaminopurine	P.O. 2,6-Diaminopurine	
	P.O. 1-Methyl-4-aminopyrazolo-(3,4-*d*)-pyrimidine	I.P. 1-Methyl-4-aminopyrazolo-(3,4-*d*)-pyrimidine	
	I.P. Actinomycin J	P.O. Actinomycin J	
		I.P. Cinerubin A	P.O. Cinerubin A
		P.O. ω-[N,N-Di-(2-chloroethyl)-p-aminophenyl]butyric acid (CB 1348)	I.P. ω-[N,N-Di-(2-chloroethyl)-p-aminophenyl]butyric acid (CB 1348)
		P.O. N,N-Di-(2-chloroethyl)-p-aminophenylalanine (CB 3025)	I.P. N,N-Di-(2-chloroethyl)-p-aminophenylalanine (CB 3025)
		I.P. TEPA	P.O. TEPA
	I.P. Demecolcine	P.O. Demecolcine	

* The letters P.O. signify oral administration, the letters I.P. signify intraperitoneal administration.

TABLE 4

Type 1	Type 2	Type 3	Type 4
		Azaserine	
		6-Diazo-5-oxo-L-norleucine (DON)	
8-Azaguanine		6-Mercaptopurine (6-MP)	
Adenine			
2-Thiouracil		5-Fluorouracil	
		DL-Ethionine	
Urethan			
		Actinomycin C	
		Actinomycin D	
		Nitrogen mustard	
		Nitromin	
		TEM	
		2,5-Bis-ethyleniminobenzoquinone	
		2,5-Bis-ethyleniminohydroquinone	
		2,5-Bis-acetamino-3,6-bis-ethyleniminobenzoquinone	
		2,5-Bis-propoxy-3,6-bis-ethyleniminobenzoquinone (E 39)	
		Myleran	
		Colchicine	
	Cortisone		

between the tumor-bearing and normal animals 'could reasonably' be due to a modification of the toxicity of the administered compound following reaction with a tumor component." This has also been noted in our experiments, for example, with 1-methyl-4-aminopyrazolo-(3,4-*d*)pyrimidine, cinerubin A, and other substances in which, after intraperitoneal administration, the LD_{50} value for normal animals is lower than that for those with tumors. One compound, cortisone, and probably also other anti-inflammatory corticoids, inhibit only granuloma growth.

It is surprising that compounds that had already proved effective to some extent in clinical use displayed no growth-inhibiting activity in these experiments. It must be pointed out, however, that 8-azaguanine, adenine, 2-thiouracil, and urethan were administered only up to a maximum dose of 300 mg./kg. without in every case reaching the maximum tolerated dose.

No compound has yet been found that inhibits only tumor growth and not granuloma growth, thereby fulfilling what are presumably the requirements for a specific tumor-inhibiting agent.

It is clear, of course, that the results and considerations outlined here apply only in the experimental framework under discussion; in particular, comparisons between effects on the tumors and on the granuloma are valid only as far as the three tumors used by us are concerned. In other experiments, for example, with 8-azaguanine, which is known to exert a significant inhibiting effect on adenocarcinoma EO 771, other conclusions must be drawn, also taking into account the details of the recent results of Dunning[20] and Stock and Sugiura.[21] At present, the experimental setup as described seems likely to make possible the confirmation of the intrinsic value of new tumor-inhibiting chemotherapeutic agents that may be developed and to facilitate the discovery of new correlations between experimental results and clinical observation, thereby contributing to the progress of both empirical and rational approaches.

Summary

A comparative method of characterizing the specificity of action of possible tumor-inhibiting compounds has been described in which three transplantable malignant tumors are used in rats, as compared with nonmalignant rapid-growing tissue in the form of a foreign-body granuloma. The method is illustrated by reference to experiments with 30 compounds belonging to different chemical groups, some of which have already been employed clinically.

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Discussion of the Paper

Murray J. Shear (*National Cancer Institute, Public Health Service, Bethesda, Md.*): Apart from the profound differences among many types of tumors or even the same tumor in the same individual at a different time, it is important that we consider the specificity of action. After all, all malignant tissue could be destroyed very simply with potassium cyanide—if we had no objection to destroying all nonmalignant tissue with it.

Joseph H. Burchenal (*Sloan-Kettering Institute for Cancer Research, New York, N. Y.*): There is a certain specificity of action any time we inhibit a tumor or increase the longevity of an intact animal with a tumor or a leukemia, because the important normal tissues in the area are limitations. With our patients, we do not really care what normal tissues are damaged by the drug if they are not vital tissues. The limiting factor in chemotherapy is the effect on the bone marrow, on the gastrointestinal mucosa, on the liver, or on the kidney—thus a certain amount of specificity is required.

Shear: True. However, does my agreement then lead to the conclusion that microbiological systems or tissue culture are useless for anticancer investigations?

Burchenal: Far from it. However, I think one must keep in mind that if there is no control of normal tissue of some sort perhaps the systems do not mean as much as studies in the intact animal insofar as relation to the patient is concerned.

Shear: I think that should be the conclusion drawn. Whether we all like to work with bacteria or tissue culture, however, does not mean our work is going to be useful. The danger is that what one group uses in this general field may be misinterpreted to mean that the operators are guaranteeing that that particular procedure will identify clinically useful drugs. So many investigators are being drawn into the field, however, that the various facts and facets that receive attention should accumulate and ultimately bring us closer to our goal.

These are important points, but I should like to get back to Loustalot's proposal of using this kind of nonmalignant proliferation for comparison of the effect of chemotherapeutic agents with their effect on malignant tumor.

Loustalot: It is obvious that one cannot generalize from this method. We have chosen this system because it is easy and gives rapid quantitative results that are reproducible. To date, the results obtained have been negative inasmuch as no specific tumor-inhibiting compound has yet been characterized. However, in a sense this speaks for the validity of our system, since the experimental results that it has yielded are in line with the clinical observation that no completely specific tumor-inhibiting compound has yet been found. Should clinically useful compounds be successfully developed, the future will show whether or not our experimental tumor/granuloma system matches the clinical findings.

TRIAL OF CHEMOTHERAPEUTIC AGENTS IN SPONTANEOUS TUMORS IN DOGS

By John R. McCoy

Bureau of Biological Research, Rutgers University, New Brunswick, N. J.

The dog develops cancer with approximately one half the frequency of man and most frequently of all the domesticated animals. It is one of the few companions of man that is permitted to live out its life span in essentially the same environment as that of its master.

The position of the dog is unique: as a rule this animal in the United States is present within a human family group as a pet sharing a dietary regime similar to that of the human, which mirrors to some degree the economic and nationalistic factors that govern the selection of foods by a given family.

Similarly, the development of this relationship has caused the rise of a facet of veterinary medical practice devoted to control of the diseases of the dog. Enlightened use of these professional facilities has led to the study of this animal at various ages during its life span and, mainly at veterinary colleges, to its clinical examination, and to necropsy for the purpose of discovering what disease has terminated its life span. Research on combatting such diseases as distemper, which have caused large losses in young dogs, and on nutritional problems has resulted in the attainment by greater numbers of these animals of their "cancer age." This has revealed a definite tendency for the canine to develop neoplastic diseases, beginning in its seventh year.

The incidence of neoplasia in the dog is probably about 10 per cent. The most frequent cases are those of the mammary gland, skin and adnexae, such as sweat glands and sebaceous glands; and basal cell, squamous cell, and epidermoid carcinoma. Mast cell sarcoma, fibrosarcoma, neurofibrosarcoma, osteosarcoma, hemangiosarcoma, and malignant melanoma are common. Sustentacular cell tumor and seminoma of the testis are frequently seen, but teratomas are rare. Malignant lymphoma, both lymphoblastic and lymphocytic, is frequently seen.

Neoplasms notable by their paucity among dogs in contrast with man are chorionepithelioma, mesothelioma, and tumors of the heart, spleen, gall bladder, and the genitourinary, gastrointestinal, respiratory, and central nervous systems, as well as myeloid leukemia.[1-3]

The preponderance of the dog's neoplasms are, therefore, in surface regions readily available for direct measurement of gross size and for repeated biopsies.

The dog may be studied under rigid environmental control in the laboratory, housed in metabolism cages, and fed semipurified diets of known composition and biological value. This permits the study of the tumor-free dog for a comparative measure of the effect of the tumor on the host, or to duplicate protein depletion, for instance, in order to study the effect of this on the dosage range for a given chemotherapeutic agent.[4]

Such measures may be conducted over protracted periods of time in the same animal, depending on the nature of the neoplastic disease present. Thus, a case of malignant lymphoma may live for only 1 to 4 weeks untreated, or more than 14 months when treated with an effective agent such as 2,4,6-triethylenimino-s-triazine (TEM) or *N*-(3-oxapentamethylene)-*N*′, *N*″-diethylenephosphoramide (MEPA). Other neoplasms lend themselves to prolonged study, such as the mammary cancer group, seminoma, sustentacular cell tumor, fibrosarcoma, malignant melanoma, and mast cell sarcoma; these may run a course measured in years.

Concurrent with observations on the effect of the chemotherapeutic agent upon the visible evidence of neoplasia represented by the tumor, measurements of other factors are possible, such as exploring methods of administration of the agent under study in order to determine the level of maximum therapeutic effect and the maximum total dose tolerated. In our studies at the Bureau of Biological Research, the dividing line between therapeutic and toxic doses fell within the range of 1.5 to 1 mg. of MEPA/kg. of body weight given intravenously for 3 successive days, followed by 4 days' withdrawal; orally, between 3 and 2 mg. of MEPA/kg. No standard total dose limit was reached, although more than 4 gm. was given to one patient with seminoma (CR105) before death.[4] According to physicians using this agent in human neoplasia, the maximum total dose tolerated by humans appears to be 700 mg.

The degree of therapeutic effectiveness against a certain group of neoplasms and the influence of continued administration on different body systems may be studied, perhaps for several years, as in a case of gingival fibrosarcoma (CR30), which was on a schedule of TEM for several months and on MEPA for more than four years.[4]

At the same time the effect of the physiological status of the host in terms of resistance to toxic effects of the agent, as well as the depleting effects of the disease, may be studied.

Efforts calculated to enhance the efficiency of this resistance by nutritional supplementation may be evaluated. Dogs fed a low-casein diet containing 0.2 gm. of nitrogen/day/kg. of body weight, supplemented with 0.67 per cent methionine and 0.67 per cent glycocyamine, did not develop leukopenia for at least 3 times the period of time that it took for leukopenia to develop in the control dogs.[4]

Ample material is available for study of the effect of the disease and the agents on the chemistry and cytology of the body fluids. The reduction of the albumin-globulin ratio in tumor-bearing dogs has been shown. The duplicating effect of this type of depletion by the administration of MEPA has been reported.[4]

There is usually material for periodic biopsies for histological evidence of antitumor effect as indicated by variation in gross size of the primary and any secondary neoplasms, making possible repeated evaluation of morphologic and histochemical changes, the latter in nucleic acid content and enzyme systems of interest.

Problems in acute toxicity may be met, means of correction may be sought, and the significance of repeated incidents in terms of effect on the host-tumor relationship may be observed. Acute MEPA toxicity, for instance, has been manifested in our canine patients by marked leukopenia and thrombocytopenia. This was followed by salivation; emesis; inappetence; pyrexia, 104° F., or higher; diarrhea, which may become bloody; lethargy; dehydration; dyspnea; limb edema; ventral edema of the head, neck, and body; interstitial hemorrhage; prostration; and death. The first 11 patients evidencing this syndrome died at varying times up to 2 weeks after the onset of symptoms. Various standard antidotes, antibiotics, fluids, and blood infusions were of no avail. Antihistamine in recommended dosage was in vain, for the patients died. The shocklike symptoms mimicked moderate anaphylactoid reaction to such a degree that it was decided to disregard warnings of possible convulsions following use of the antihistamine* under study and to give massive doses with parenteral fluids and broad-range antibiotics to control secondary infections during the leukopenic period.[4] This regime has since been successful against this event 23 out of 28 times used. Convulsions did not occur.

Further value from the use of the dog as an intermediate tool in chemotherapeutic investigation is found in considering the known disparity between the antitumor effect on the transplanted tumor and that on the spontaneous neoplasm. Except in a case of fibrosarcoma of the palate, no tumors other than the lymph nodes of dogs afflicted with malignant lymphoma have regressed completely, the effect being produced by MEPA, for instance, being at best carcinostatic for varying lengths of time. The Flexner-Jobling tumor in Sprague-Dawley rats regressed in 80 per cent of the test animals treated with MEPA.[5] The Walker 256 and R-1 tumors did not regress unless certain dietary adjustments favoring the host's relation to its tumor were made.[6]

Differences have been observed in the behavior of certain analogous tumors in the dog and man. Mammary neoplasms of the canine do not show a comparable tendency to metastasize to the skeleton. Seminoma of the dog's testes does not evidence comparable metastatic properties.[7]

Recently we have tried certain therapeutic approaches combining pretreatment of 7 mammary neoplasms and 1 sudoriferous adenocarcinoma with MEPA for 3 to 6 weeks, at which time the neoplasm, although it may be evidencing growth, becomes surrounded by an areolar connective tissue, permitting surgical removal. The mammary tumors became pendulous, hanging down from the belly wall so that the skin could be pinched between the 2 structures. This is considered the indicator for surgical intervention, as noticed on necropsy of 2 patients with recurrent mammary neoplasms early in our studies and reported previously.[8]

Further investigations in combined therapy are contemplated by us, including nutritional and hormonal, as well as the chemotherapeutic and surgical studies of this disease in the dog.

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Discussion of the Paper

Murray J. Shear (*National Cancer Institute, Public Health Service, Bethesda, Md.*): This report should open our eyes to a really important matter. One of the encouraging things about the work as presented here is that it is not only aimed directly at chemotherapy, but also has involved some investigation without thought of chemotherapy that may have some pertinence to it. One of the great needs in this field is more information about what happens to drugs when they are given for treatment of cancer. What are the blood levels? Do they go up or down? Do they rise and remain high for a certain period? How are they excreted? How are they metabolized? The dog, as we have just learned, provides a wonderful opportunity for learning these things because it is much better than the mouse or the rat in regard to providing successive blood samples and successive biopsies and metabolism experiments. Further work in that direction should be well repaid.

Comment: I have heard that tumors of dogs are quite variable. Would McCoy comment on this aspect?

McCoy: Within diagnostic realms I think tumors of dogs can be pretty well categorized. The mixed mammary tumor, for instance, can sometimes be studied for 2 and 3 to 5 years. Generally this tumor is thought to be related to estrogen levels in the dog; this is accepted at least clinically, because they respond to androgens. I do not believe any really critical study has been made of the endocrinology of this, however, although certainly we hope it will be studied. The infiltrating papillary duct adenocarcinoma will go to perhaps 2 years at most. The so-called "alveolar" or solid adenocarcinoma may kill as early as 3 weeks.

In malignant lymphoma there is more variation based entirely, I believe, on the nutritional status of the dog. If the animal has not been on a reasonably good diet it will not stand up to this disease. It usually dies, perhaps 2 weeks after the lymph node swelling has become apparent. We have studied cases without treatment where, on a high protein diet, the dog has gone as long as 4 months. We have never seen a dog go to 7 months, as claimed in the literature. There are case reports of dogs going 5 years many years ago in Germany, but we feel these may not have had the benefit of microscopic diagnosis.

Shear: It cannot be denied that dogs, as presently available, are not homozygous. We cannot get dogs in groups of 25 each and transplant tumors and have them take and have them grow at 14 or 17 days, and have 25 of the same size controls as the 25 used for treatment. Furthermore, I should certainly not suggest that we abandon work with the rodents. The additional work with dogs, however, should well reward us for the effort. The fact that dogs are variable might be an advantage in that it more closely approximates the clinical situation. Also, as McCoy has said, it is permissible, according to our ethics, to do work for the benefit of the dog that might occasion criticism if done for the benefit of man.

PRACTICAL AND THEORETICAL CONSIDERATIONS IN THE USE OF INDUCED AND SPONTANEOUS MAMMARY TUMORS IN CANCER CHEMOTHERAPY*

By Jean Scholler

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It is the purpose of this paper to present some of the problems inherent in the use of spontaneous or 7,10-dimethylbenz[α]anthracene (DMBA) induced rodent mammary adenocarcinomas for cancer chemotherapy screening. Throughout this monograph great emphasis has been placed on the correlation of results obtained with the tissue culture, microbiological, and other diverse systems with results obtained from long-established transplanted tumor systems. The ease of handling, the relatively low cost, and the rapidity with which results are obtained are a few of the points discussed for the various systems under consideration. Unfortunately the spontaneous mouse or induced rat tumors that my associates and I have studied offer none of these advantages. Details of the procedures employed for the spontaneous mouse tumors have been described previously[1-4]. The procedure of Geyer *et. al.*[5] has been employed for the induction of mammary tumors in the rat with DMBA. Our preliminary results also have been described.[6]

In recent years interest has been aroused in the possible use of spontaneous or induced rodent tumors for cancer chemotherapy. It is well known that the agents that are effective against transplanted tumors are for the most part ineffective against spontaneous tumors. These same agents have shown little if any effect against the solid tumors of man.[7] At present no satisfactory explanation can be given for these apparent discrepancies. Since the purpose of this publication is to evaluate critically the various methods under discussion, the results of chemotherapeutic studies will not be given, except as they are pertinent to the discussion. Instead, I propose to discuss several practical and theoretical problems involved in the use of spontaneous or induced tumors for chemotherapy studies.

One of the primary considerations is the availability of mice bearing spontaneous tumors. At present there is no source of large numbers of these animals. We have had to depend on J. J. Bittner of the University of Minnesota, Minneapolis, Minn., and on the Roscoe B. Jackson Memorial Laboratories, Bar Harbor, Me. The total numbers available have averaged approximately 100 per week, at a cost of between \$3 and \$5 per mouse, plus the expense of air shipment. Also, it should be kept in mind that only 50 to 60 per cent of the animals received are suitable for chemotherapy trials. This is due to several factors. First, as most tumor-bearing mice are old breeders, there are always some pregnant animals in any shipment. These cannot be used until after parturition and, in many instances, by this time their tumors are too large for use in the therapy trials. Second, the sources mentioned are not engaged primarily in producing spontaneous tumor-bearing mice for commercial purposes. Therefore all animals with palpable masses are shipped as soon as noted. Thus, after holding the mice for a week before setting up therapy trials, many of the masses regress or fail to grow or appear cystic in nature. These are discarded. Finally, in our studies we have selected arbitrarily a range of 5 to 15 mm. diameter as the limit for tumor size at the start of an experiment and, during the period of observation, some of the tumors grow rapidly to a size too large for use.

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At the present time, it would be necessary for those interested in setting up a chemotherapy program using spontaneous mammary mouse tumors to produce their own tumor-bearing animals. With this thought in mind, the data in TABLE 1 are presented. This table was compiled after a discussion with Bittner. It should be noted that the total costs given for A or B do not include the cost of providing space to maintain this breeding colony, which would vary greatly, of course, depending upon the space available in existing buildings. It is by no means a small or inexpensive undertaking even to produce sufficient mice for only several experiments per week. The expense of producing two hundred rats per week bearing DMBA-induced mammary tumors is equally great. Another point that should be mentioned is that the cost of carrying out chemotherapeutic trials in a program such as we are discussing is also considerably more than that incurred with the long-established transplanted tumors.

All animals are observed for one week before the start of therapy. Because of the wide variation in tumor size, the use of mean values with standard deviations is not feasible Therefore, we have converted tumor size to growth index and have employed the Rank t test[8] to determine the significance

TABLE 1

CONSIDERATIONS INVOLVED IN SETTING UP AND MAINTAINING A COLONY TO PRODUCE 200 SPONTANEOUS TUMOR-BEARING MICE PER WEEK

Requirements/year	Cost/year
A. Minimum requirements per year	
Breeding colony—to produce 300 ♀/week	$ 2,000
Space and maintenance for 15,000 mice	54,000
Two technical assistants	7,500
Total	$63,000
B. Requirements per year if animals are bred	
Two additional assistants	$ 7,500
Three additional animal caretakers	8,000
Total	$78,500
C. Initial requirements	
Cages for A 3,000	$15,000
Cages for B 8,000	40,000
Space	?

of differences in tumor sizes. Growth index is herein defined as the value obtained by comparison of individual tumor size at any given time with its size at the beginning of an experiment. Thus all tumors have a growth index of 1 at 0 day. It should be mentioned that growth index is based on the calculated volumes of the tumors, derived from the mean diameter of the tumor (based on the assumption that the tumors are spherical).[1]

This now brings us to the third problem, variations in the growth rate and survival time of spontaneous tumor-bearing mice. FIGURE 1 shows the comparative rates of growth, expressed as growth index, of first-generation transplants, of spontaneous mouse tumors, and of DMBA-induced mammary tumors of the rat. These values are derived from groups of 200 to 300 control animals for each system. The transplants, only 1 generation removed from the spontaneous tumors, increase over one hundredfold in size during the 21-day period. On the other hand, the spontaneous mouse and induced rat tumors show only an average sixfold to sevenfold increase in size during the same period. The variations within the latter groups range from a growth index of less than 1 to more than 100. The mean survival time of 300 mice bearing transplants was 33 ± 5 days while, in a control group of 300 mice bearing spontaneous tumors, the mean survival time was 47 ± 20 days, with a range of 5 to more than 100 days. The mean survival time for rats bearing induced tumors is difficult to evaluate, since only 60 per cent of the animals die within 4 months after the appearance of tumors. Of the remaining 40 per cent, only a few die in the next several months. We have not maintained large numbers of the rats for a sufficiently long period to ascertain the range of time of death.

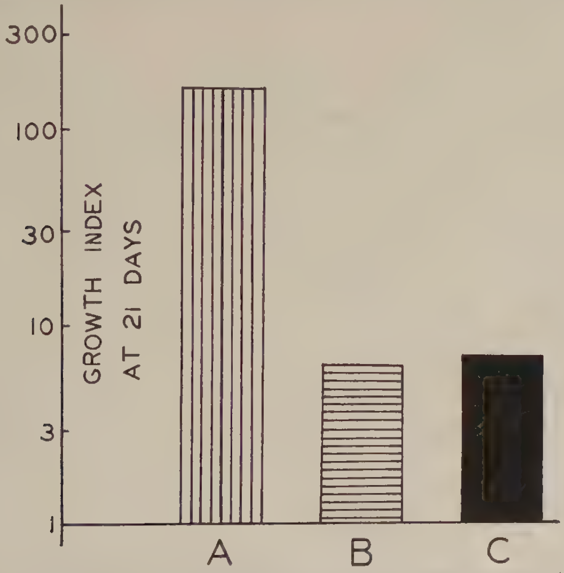

FIGURE 1. Comparative rates of growth expressed as growth index of (A) first-generation transplants, (B) spontaneous mouse tumors, and (C) DMBA-induced mammary tumors of the rat.

Let us turn now to several of the more theoretical considerations in the use of these spontaneous and induced tumors for chemotherapeutic studies. When these studies were initiated, two possibilities as to the usefulness of these systems seemed probable. One or both might be employed as an adjunct to a primary screening procedure or as a specific tool in the evaluation of the hormones. The adjunct screen would provide additional information for a specific compound or group of compounds as an aid in the selection of compounds for clinical trial. On the other hand, the hormonal response of human mammary cancers is well known. If these tumors could be shown to be hormone-dependent, this would provide a useful tool for the evaluation of the many new estrogens, anti-estrogens, and androgens that now must wait for clinical evaluation as to their effectiveness in mammary tumors.

Our attention was first focused on possibilities in the use of spontaneous mouse tumors as an adjunct screening procedure. TABLE 2 presents the results obtained using the agents found to be highly active in one or more long-established transplanted tumor systems.[2-4] All compounds tested were either ineffective (negative) in inhibiting the growth of the spontaneous tumors or were effective (±) only at doses that caused severe weight loss or fatalities or both. The combination of ARDMA and STEPA effectively inhibited the growth of the tumors and, although weight loss was evident during treatment, produced a significant increase in survival time. Several compounds that have shown little or no activity against transplanted tumors also were investigated against

TABLE 2

RESULTS WITH SPONTANEOUS MAMMARY TUMORS IN MICE

	Inhibitory		
Negative	With toxicity		Without toxicity
	No increased survival time	Increased survival time	Increased survival time
(−)	(±)	(+)	(++)
STEPA	DON	ARDMA + STEPA (1/5 trials)	
Triazene	Nonane		
6-Mercaptopurine	TEPA		
Ribonuclease	Thiocolchicine		
DMAP	4-APP		
Styrylquinoline	1-Me-4-APP		
Mitomycin	ARDMA		
Fumagillin			
Hydrocortisone			
Estradiol			
Stilbestrol			
Progesterone			
Testosterone			

Abbreviations: STEPA, N,N',N''-triethylenethiophosphoramide; triazene, 3,3-dimethyl-1-phenyltriazene; DMAP, 1,2-dimethyl-4-amino-5-hydroxybenzene; styrylquinoline, 4-(*p*-dimethylaminostyryl)quinoline methiodide; DON, 6-diazo-5-oxo-L-norleucine; nonane, 1,9-di(methanesulfonoxy)nonane; TEPA, N,N',N''-triethylenephosphoramide; thiocolchicine, *N*-desacetylthiocolchicine; 4-APP, 4-aminopyrazolo-(3,4-*d*)pyrimidine; 1-Me-4-APP, 1-methyl-4-aminopyrazolo-(3,4-*d*)pyrimidine; ARDMA, the aminonucleoside of Puromycin, 6-dimethylamino-9-(3′-deoxy-3′-amino-β-D-ribofuranosyl)purine.

TABLE 3

COMPARATIVE EVALUATION OF VARIOUS TYPES OF MAMMARY ADENOCARCINOMAS

	Human	Spontaneous mouse	Induced rat	Transplanted mouse or rat
Hormonal dependency	Yes	No	No	No
Mestastases				
Lung	Yes	No	No	No
Bone	Yes	No	No	No
Chemotherapeutic response*	Neg.	Neg.	Neg.	Pos.

* To agents other than hormones.

the spontaneous tumors. Again no activity was found. Because of the lack of responsiveness of the spontaneous tumors to any of the many compounds, its usefulness as an adjunct screen at the present time is doubtful. However, a much larger series of compounds of varying type structures and activities must be tested before the spontaneous tumors are completely discarded in this capacity.

The question of hormonal dependency is the last but by no means the least consideration. Estradiol, stilbestrol, progesterone, and testosterone, as well as combinations of estradiol and progesterone, have been tested in both the spontaneous mouse tumors and the DMBA-induced rat tumors.[9, 10] At the physiological and pharmacological doses examined, neither system has responded. The growth of the tumors was not affected; neither inhibition nor stimulation of growth was seen. The effects of pregnancy also were studied. There appeared to be no effect on the growth of the DMBA-induced rat mammary tumor either during pregnancy or during lactation.[10] Again, further studies must be carried out on the effects of castration, adrenalectomy, and hypophysectomy. However, it appears that these tumors will offer little advantage over the established transplanted tumors for the study of the effects of hormonal environment on the growth of rodent mammary tumors (TABLE 3).

In conclusion, it should be emphasized again that more extensive studies are needed to complete the evaluation of the usefulness of spontaneous mouse mammary tumors and DMBA-induced rat mammary tumors for chemotherapeutic investigations. Although their response to agents known to inhibit the growth of transplanted tumors is not striking, they may be useful perhaps in the screening of a series of structurally related compounds in an effort to help the clinician decide which one of the series might be the most useful in human trials. Although it appears that these two systems are not truly hormone dependent, further work is required in this direction in an attempt to understand the mechanisms involved.

Acknowledgments

I express appreciation to J. J. Bittner and Nathan Kaliss for cooperation in making available spontaneous tumor-bearing mice, to S. S. Sternberg and R. E. Carnes for histological diagnoses of the tumors, and to F. S. Philips for encouragement and advice.

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Discussion of the Paper

Murray J. Shear (*National Cancer Institute, Public Health Service, Bethesda, Md.*): Species differences are certainly an important aspect of this work. Also, it does no harm to repeat that secondary tumors may behave differently than spontaneous or induced primary tumors. In fact, I hear reports that, in patients with more than one tumor who are given a chemotherapeutic agent, one tumor will regress and the other may not. Ultimately, it may come to something like "custom-tailored" chemotherapy in which, in each patient, a biopsy will be taken not only for the pathologist but for the chemotherapy laboratory, so that the laboratory can run through ingenious procedures not yet developed to discover which agent is best for that particular tumor in that particular patient. If all tumors do not regress, the metastases will be biopsied and run through the same process. Obviously, screening directly in patients is surrounded with far too many difficulties to be practicable.

Comment: I think it was said that it is unusual for human breast cancer to be multiple primaries at the same time. Edward Gaul, at the University of Cinncinati, has studied this recently with large sections through breast cancer, and he finds quite commonly that human breast cancer has multiple foci of origin at the same time in the same breast.

Scholler: Certainly more work should be done on development of the methodology, perhaps on new tumor systems that may prove more useful than those already existing.

POSSIBLE UTILITY OF THE ROUS SARCOMA FOR ANTITUMOR SCREENING

By Irving S. Johnson, Linville A. Baker, and Howard F. Wright
Biological Research Division, Eli Lilly and Company, Indianapolis, Ind.

This paper is an interim report on a study that is still in progress. Previous reports[1,2] have described the utility of a Rous sarcoma system in the detection of antiviral activity of a highly selected group of antibiotic broths as correlated with concurrent tests in a mammalian virus system. In this test 0.2 ml. of arbitrarily selected log dilutions of semipurified Rous sarcoma virus (RSV)* in tissue culture medium 199 containing 10 per cent inactivated horse serum are injected between the 2 layers of skin of the wing web of groups of 14-day-old White Leghorn chicks. This infection results in a localized wing tumor which, if allowed to go unchecked, metastasizes, killing the host, but may be modified by intraperitoneal treatment of the host with various agents. These tumors are easily removed at the end of the experiment and may be weighed to obtain mean tumor weights per bird for comparison with those of saline- or water-treated controls. Such comparisons in the case of the group of highly selected antiviral antibiotic broths resulted in dose-response curves and highly reproducible effects; in some cases these approximated 100 per cent inhibition of the tumors.

In the present study a second group of antibiotic broths was selected on the basis of possible activity against transplantable murine neoplasms. These broths have given a 30 per cent or greater inhibition of Sarcoma 180; prolonged life by 20 per cent or more when tested against the P 1534 leukemia; or, in a few rare instances, proved active against both murine tumors. An important difference should be borne in mind concerning the effectiveness of the selection systems for the antiviral and antitumor broths. In the former case, the antiviral broths had repeatedly given evidence of activity against a mammalian virus. The antitumor broths, on the other hand, had been for the most part of a low order of reproducibility when they were at all reproducible, and the effects had never been more than minimal. We did not expect to obtain the same degree of correlation of activity or magnitude of response between the RSV and mouse tumor systems as was obtained between the RSV and mammalian virus systems. We did feel that concurrent tests of the same fermentation lots of these broths in the RSV and murine tumor systems might furnish a preliminary evaluation of the utility of the RSV as a screening tool for agents possessing potential chemotherapeutic activity in experimental malignancy.

A far greater number of the broths tested significantly inhibited the Rous tumor, that is, by more than 35 per cent, than did either of the mouse tumors when tested concurrently (TABLE 1). Slightly more than 50 per cent of the broths were positive by our standards in the Rous test, while approximately 10 per cent still indicated activity in the murine systems. These data may well

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* Lot CT694 of this virus was prepared and supplied to us by W. Ray Bryan of the National Cancer Institute, Public Health Service, Bethesda, Md.

TABLE 1

Initially active on	No. of broths	Concurrent test against					
		S 180		P 1534		RSV	
		Pos.	Neg.	Pos.	Neg.	Pos.	Neg.
S 180 only	25	3	22	0	25	14	11
P 1534 only	7	1	6	2	5	4	3
Both	3	0	3	0	3	2	1

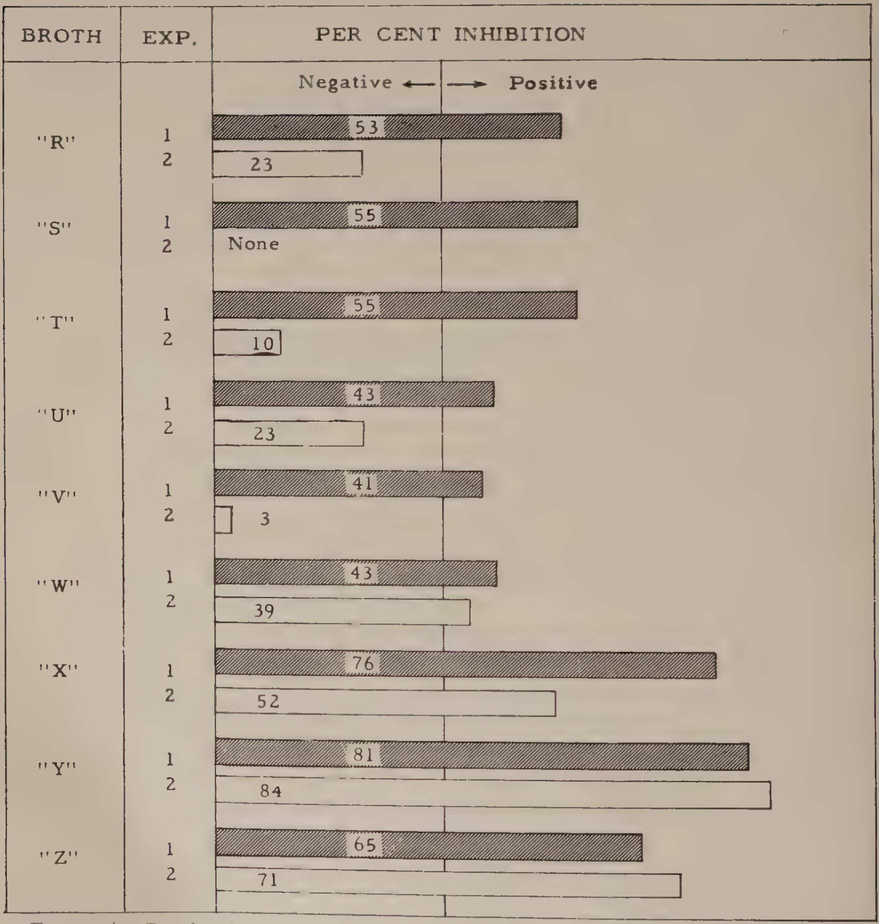

FIGURE 1. Results obtained in checking the reproducibility of both the fermentation and test systems.

point out a potential fault in the RSV system, namely, that of being "too" sensitive or of producing so-called false positives. A second interpretation that we felt to be justified in the study with the antiviral broths is that the RSV system is actually more sensitive and reproducible. The results of further work are necessary for any definite decision on this matter.

Six of the antitumor broths that were only moderately or slightly active in their initial RSV test and 3 of the more active broths were regrown and retested as a challenge of the reproducibility of both the fermentation and test systems (FIGURE 1). Only 1 of the 6 moderately or slightly active broths significantly inhibited the Rous tumor in the repeat test. From our experience with organic compounds and antiviral broths in the Rous test and antitumor broths in murine tumor systems, we feel that the failures in the repeat test were probably failures in the reproducibility of the fermentation. All 3 of the more active broths, X, Y, and Z, however, repeated both in fermentation and significance of tumor inhibition. These data suggest that repeat tests would eliminate the false-positive broths that cannot be depended upon for reproducible fermentations. All active broths have been reproducible when the same lot has been used in the repeat tests. A significant dose-response curve was obtained with broth Y (FIGURE 2). A similar quantitative response

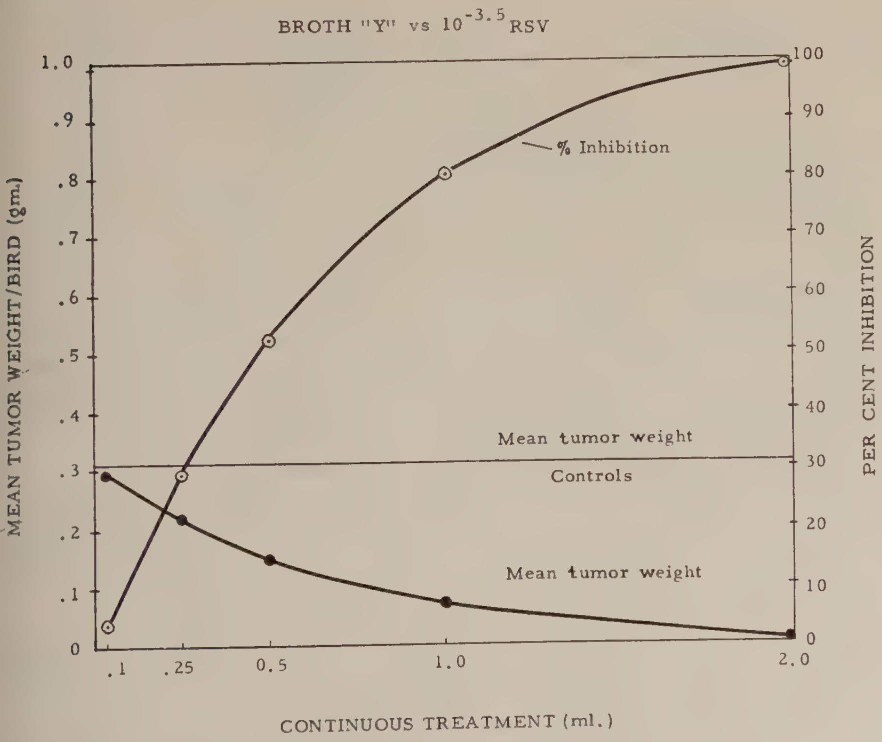

FIGURE 2. Dose response curve obtained with antitumor broth Y.

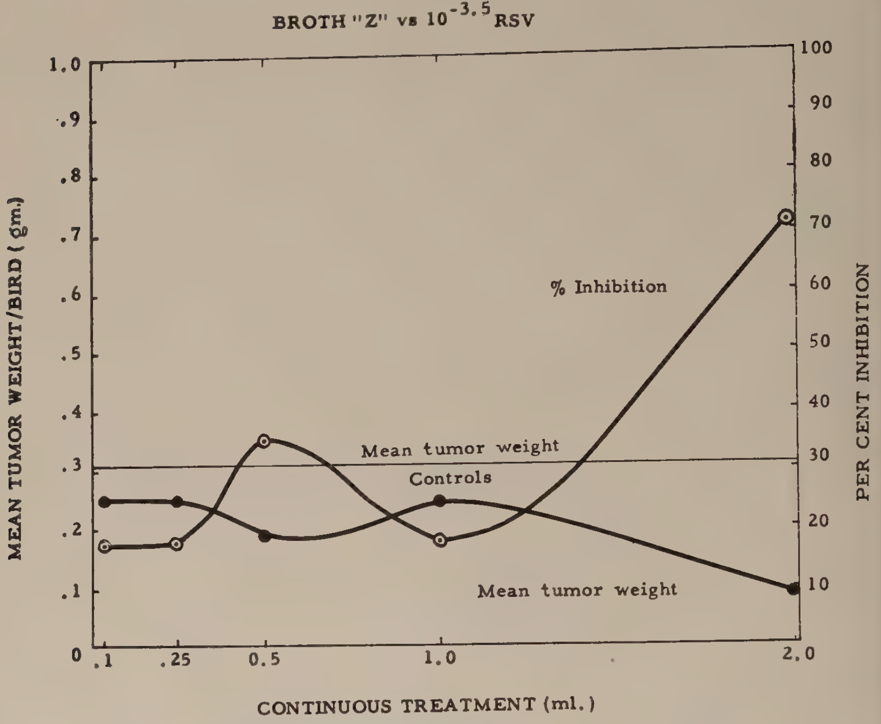

FIGURE 3. Dose response curve obtained with antitumor broth Z.

was not obtained with the less effective broth Z (FIGURE 3). Active lots of the other broths have not yet been studied in this manner.

A representative but by no means all-inclusive group of known antibiotics and antitumor compounds has been tested against the Rous tumor (FIGURE 4). Actinomycin was the only one of 8 antibiotics to give a significant inhibition of the tumor. The results obtained with various known antitumor drugs suggest that the RSV system is not as sensitive to alkylating agents as are drugs of the antimetabolite type. All the drugs retested have resulted in tumor inhibitions within 10 per cent of the initial figure. One interesting experiment in this group illustrates the ability of this system to distinguish an active antitumor compound, azaserine, from a structurally related weaker drug, γ-glutamylhydrazide, and the metabolite they inhibit, L-glutamine.

A few reproducible experiments have been performed with zymosan in the RSV system (FIGURE 5). The significance of these results is not clear, and interpretation is necessarily speculative. A single administration of zymosan 24 hours prior to infection with RSV gives a significant inhibition of the tumor, while a single injection of zymosan 24 hours following RSV infection has almost no significant effect. Theoretically, the former result could be ascribed to the heightened properdin "rebound" effect, which has been described in mammals, and the latter result to the infection being active prior to any such

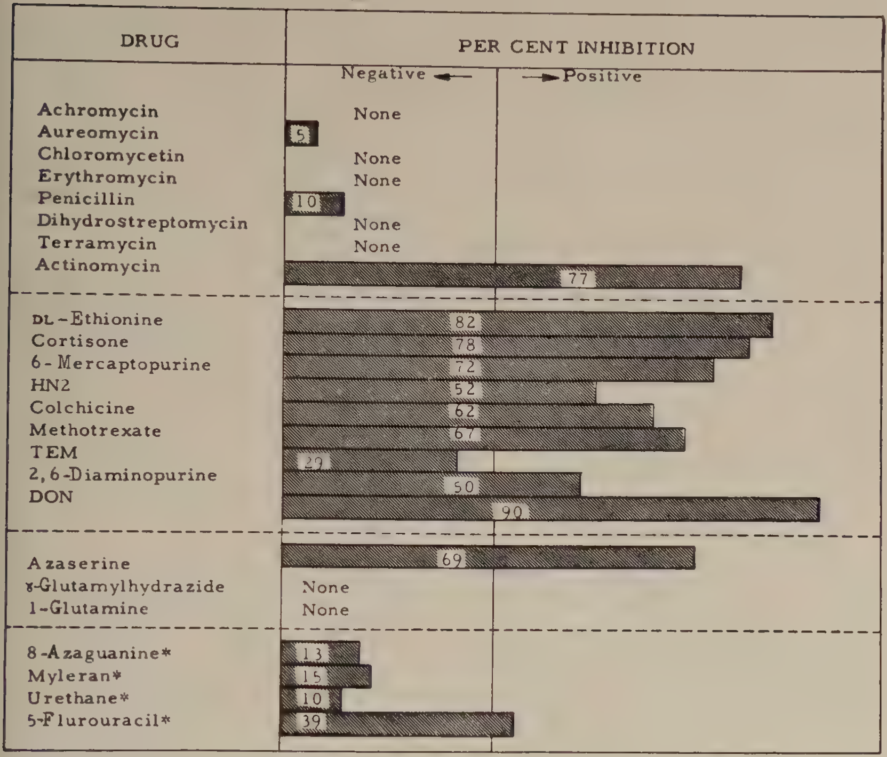

FIGURE 4. The effect of various antibiotics and certain known antitumor drugs on the RSV tumor. Drugs followed by an asterisk were tested only once. Gains in body weight indicate that they should be retested at higher levels, which may result in some positive response.

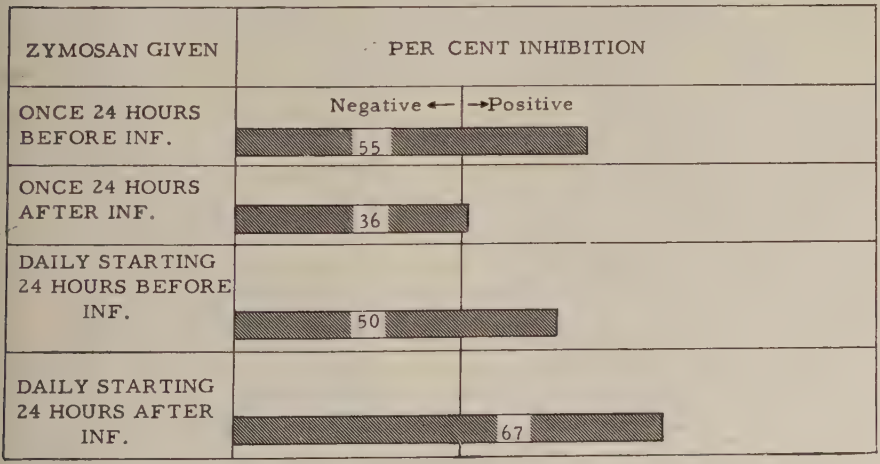

FIGURE 5. The effect of zymosan on tumors resulting from a 10^{-3} RSV infection.

rebound effect. If properdin has an inhibitory effect in this malignancy, however, continuous administration should maintain a low properdin level, resulting in uninhibited tumor growth. Continuous daily administration of zymosan actually results in an increased antitumor effect in the RSV system. These data are difficult to interpret unless the zymosan-properdin relationship in avian systems is quite different from that in mammalian systems.

Two aspects of using the RSV for screening purposes should be acknowledged. Since the 14-day-old White Leghorn chick is a larger animal than the mouse, it has the disadvantage of requiring significantly larger amounts of materials for testing by daily administration. This may be alleviated, in the case of organic compounds at least, by the fact that only a slightly lesser effect is obtained by daily administration of a drug such as 6-mercaptopurine (6-MP) for 5 days than results from a daily dosage for 10 days, the usual method in this test. In the case of antibiotic broths the amount of material required is usually not a serious problem. The second aspect is the effect of known drugs and antibiotic broths of unknown constituents on the increase in body weight of the host. In this case, the host animal usually doubles in total body weight during the 7 to 10 days of the test. This usually means an increase from approximately 100 to approximately 200 gm. Almost without exception, materials that actively inhibit the tumor also partially inhibit the normal gain in weight. Such a system is advantageous because it facilitates differentiation among general mitotic poisons such as colchicine, noxious materials that inhibit appetite, antimetabolites, and antibiotic substances that inhibit either the virus infection or the tumor growth with little or no significant effect on the rapidly dividing normal cells, as is represented by the doubling in body weight of the nondrug-treated host (FIGURE 6).

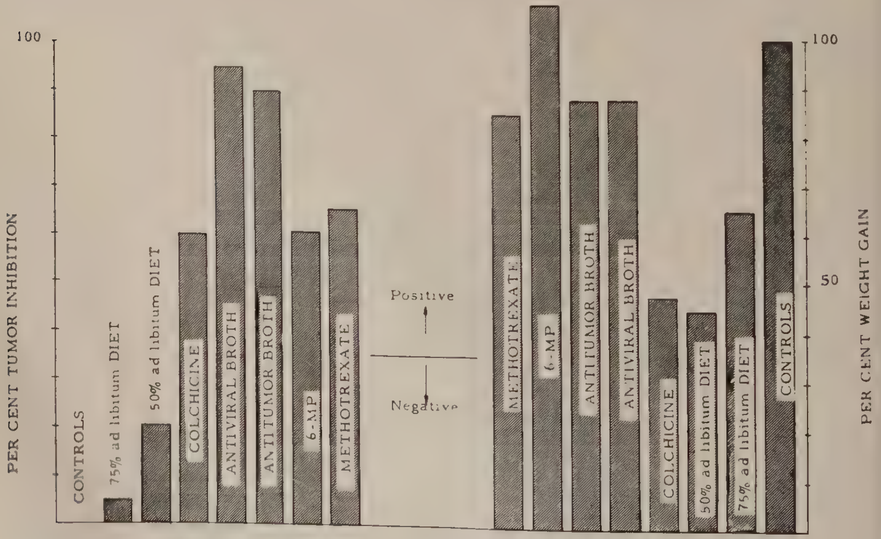

FIGURE 6. The effects of toxic drugs, antibiotic broths, and near-fatal starvation on the percentage of increase of body weight and of inhibition on the RSV-induced tumor.

The oncogenic virus system described here and the inhibitory activity of a highly selected group of antibiotic broths, as well as certain known antitumor compounds, may prove to be of only temporary interest. Much further work is required to ascertain the usefulness of this system. Nevertheless, the capacity to immobilize and/or prevent the spread of a rapidly metastasizing tumor, which is, in addition, an active virus infection, is a type of biological activity that is not routinely seen in either virus or cancer research. The demonstration of these effects and the preliminary prospect of being able to discriminate between structurally related inactive and active antitumor compounds and gross cellular poisons holds, at least in theory, new and interesting possibilities for the therapy of experimental cancer and virus infections by means of this investigative tool.

References

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Discussion of the Paper

Vincent Groupé (*Institute of Microbiology, Rutgers, The State University, New Brunswick, N. J.*): During the past few years we have also used virus-induced Rous sarcoma in studies on experimental chemotherapy in our laboratory. Our procedure differed somewhat from that used by Johnson; it utilized the latent period for tumor production in the wing web developed by Bryan and his associates for quantitative bioassay of the virus. Since Loomis and Pratt have shown that tumor cells are present histologically 48 hours after injection of virus, daily intramuscular injections of candidate materials were not begun until the third day after inoculation of virus. Using White Leghorn chicks 7 days of age and approximately 100 ED_{50} of virus, grossly visible tumors about 1.0 mm. in diameter appeared in control chicks 6 to 8 days after infection. Using the procedure just described, we have tested more than 30 compounds active in other systems against virus-induced Rous sarcoma. All failed to delay the latent period when tested at barely sublethal levels. Among these were: actinomycin D, A-Methopterin, 8-azaguanine, 8-azaxanthine, azaserine, benzimidazole, 1,5-diaminobiuret, 2,6-diaminopurine, and 6-mercaptopurine. In addition, thus far 128 microbial filtrates from recently isolated actinomycetes and Gram-negative rods were tested and, of these, 4 significantly delayed the latent period. The value of any screening procedure is determined by what it produces, and in this instance it is clearly too early to arrive at any conclusions with regard to virus-induced Rous sarcoma. In conclusion, I might add that, should any preparation active in this system turn out to be antiviral rather than antitumor, I doubt that it will be discarded.

W. Ray Bryan (*National Cancer Institute, Public Health Service, Bethesda, Md.*): The data presented by Johnson, together with those obtained by Groupé and summarized by him in his discussion, demonstrate that under the controlled technical conditions described the Rous sarcoma can serve as a highly sensitive and quantitatively reproducible indicator of antitumor activity. Therefore, this virus-induced tumor of chickens is a valuable addition to the screening materials now available for the detection of antitumor activity. Some of the advantages of its use for this purpose are presented below.

Biological considerations. One of the most important advantages of virus-induced Rous sarcomas as biological indicators of antitumor activity is the fact that the tumors result entirely from proliferation of the host's own tissues, and that they therefore simulate naturally occurring, spontaneous tumors in their relation to the test host. Consequently, positive results obtained with this indicator tumor would not be expected to include agents that act through mechanisms involving reactions against foreign tissue antigens or antigens of invading bacteria or passenger viruses, a factor that complicates the results when transplanted tumors are employed.

In addition to screening for agents that are effective against Rous tumors, the same test system may be used in screening for agents capable of preventing infection by tumor viruses, as well as for those that may be capable of arresting such infections after they have taken place, but before they have eventuated in malignant transformation. Antiviral substances effective against tumor viruses could be of considerable importance to the cancer problem if an appreciable proportion of the cancers that occur spontaneously in nature have their origin in infections due to etiological agents of this type.

Technical considerations. A technical advantage of considerable importance is the fact that the test hosts for the Rous sarcoma screening system, that is, chickens, are available commerically and can be procured in fairly large numbers on relatively short notice.

A second important factor in any screening program involving animal hosts is the maintenance of adequate amounts of "seed material" for producing relatively large numbers of comparable indicator tumors at a desired time. This problem is reduced to negligible technical proportions when virus-induced Rous sarcomas are employed.

For instance, common batches of partially purified virus material comprising several hundred standard aliquots, for example, 0.15 ml. each, can be prepared by simple laboratory procedures extending over a period of only two or three days. When sealed in glass ampules, these materials can be stored in a CO_2 ice chest for several years without loss of potency. The induction of indicator tumors therefore involves simply the procedures of removing a vial from the CO_2 ice chest, thawing and diluting the sample, and inoculating chickens with the diluted virus. The indicator tumors appear within about one week in highly susceptible hosts. In both the procedure described by Johnson and that described by Groupé the desired concentration of virus for inducing indicator tumors was the concentration that could be expected to produce just a 100 per cent incidence of tumors in chickens of the strain inoculated. The appropriate dilution was 10^{-4} for the chickens employed by both Johnson and Groupé. Thus, a single aliquot of only 0.15 ml. of strong virus would make a total of 1.5 l. of the desired virus concentration. Using inocula of 0.2 ml., this would be sufficient for the injection of 7500 chickens, a considerably larger number than would ever be required at any one time in a biological laboratory. Consequently, a single frozen lot of standard virus samples would supply all the screening requirements of several large testing centers over a period of several years.

A third technical consideration of major importance was emphasized by both Johnson and Groupé; namely, the relative ease with which quantitatively reproducible data on antitumor activity can be procured with the Rous sarcoma system. The reproducibility derives largely from the use of standardized doses of virus and the resulting yield of biologically comparable indicator tumors in successive experiments extending over relatively long periods of time.

JOHNSON: Regarding the length of our treatment with chickens, ordinarily, for routine screening we start treating the day before we infect, and we treat for 10 days. Then we sacrifice the chickens, remove the tumors, and weigh both the carcasses and the tumors, with the idea of detecting both prophylactic and therapeutic activity. Once activity of any sort is proved, we go back and design experiments to locate this activity, and then treat a group starting before and a group starting afterward. For routine screening it is 10 days, daily treatment starting the day before infection.

BRYAN: These two methods are not mutually exclusive. Johnson could, if he chose, still observe his chickens daily and obtain a latent period, while Groupé, if he chose, could continue his injections longer and weigh his tumors at the end of the experiment. In other words, both of these types of observations can be obtained on the same group of animals in the same experiment at the same time.

THE USE OF ASCITES TUMORS IN SOLID AGAR FOR SCREENING OF CANCER CHEMOTHERAPEUTIC AGENTS*

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Roswell Park Memorial Institute, Buffalo, N. Y.

Introduction

Essentially every type of material that exhibits physiological activity, including metabolites and antimetabolites, is being tested for cancer chemotherapy. Substances of natural origin are also receiving attention. Because of the extensive work required in screening cancer chemotherapeutic agents with experimental animals, many investigators have attempted to determine the feasibility of *in vitro* techniques. Thus far, no *in vitro* technique has proved to be a satisfactory substitute for *in vivo* experiments. *In vitro* techniques have been unable to surmount conditions of animal experimentation such as the animal's modifying effects on a material or the toxic effects of the material itself on the animal. When used in conjunction with other types of screening methods, however, *in vitro* techniques may serve as a useful guide for selecting possible chemotherapeutic agents and also for selecting compounds structurally related to an active compound best suited for clinical trial.

Ascites tumor cells *in vitro* may be used to determine dehydrogenase inhibition and possibly general cell toxicity of potential chemotherapeutic agents. Miyamura (1956) reported the results obtained using Ehrlich ascites tumor cells with sixteen substances in a modified agar plate test. He concluded that this simple method is useful for screening anticancer substances. Recently, DiPaolo and Moore (1957) presented a series of experiments exploring the use of an ascites cell-plating technique for screening cancer chemotherapeutic agents. The use of a variety of cells, media, solvents, and indicators with a series of compounds was reported. It was concluded that not all chemicals known to have cancer chemotherapeutic value responded to this *in vitro* test. This is not surprising, since some cancer chemotherapeutic agents that do not inhibit the dehydrogenase activity of tumor cells show definite carcinolytic activity.

Other variations of this method, also based on the inhibition of dehydrogenase activity of cancer cells in agar, have been reported (Mizuno *et al.*, 1957). These authors have tested 1300 organic compounds using 2,6-dichlorophenol indophenol as an indicator of dehydrogenase inhibition of Ehrlich ascites carcinoma, Sarcoma 180 ascites, and Yoshida ascites tumor cells in agar with Hanks' solution. The compounds tested were divided into 27 groups according to the main structure or functional group of the compounds. Materials in 24 of the 27 groups were found to inhibit at least 1 of the 3 tumor lines used. The authors concluded that, with the exception of purines and pyrimidine antimetabolites which, they stated, do not diffuse so rapidly as other classes, most antitumor materials showed high activity.

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Since the report on the agar plate diffusion technique by DiPaolo and Moore (1957), this test has been adapted in this laboratory from a Petri dish to a test tube. This technique is simpler than the previous method and permits determination of test results in a shorter period. Techniques utilizing test tubes have also been reported by Yamamoto *et al.* (1956) and Arai and Suzuki (1956).

The use of a variety of ascites tumor cells in agar pour plates as well as in test tubes for screening cancer chemotherapeutic agents is discussed in this paper, with emphasis on the correlation of these results with those obtained with *in vivo* systems.

Materials and Methods

Ascites agar pour plate. The basic medium for the ascites agar plates was made as follows: peptone, 1 gm.; glucose, 0.5 gm.; sodium chloride, 0.25 gm.; hydrated sodium monohydrogen phosphate, 0.3 gm.; agar, 2 gm.; and distilled water, 100 ml. The *p*H of the medium was adjusted to 7.2. The final composition of each plate contained a mixture of 6 ml. of a basic medium, 1 ml. of heparinized rabbit plasma, and 1 ml. suspension in Tyrode's solution of 2×10^7 cells from a 7- or 8-day-old tumor inoculum. Although Ehrlich-2 ascites obtained from T. S. Hauschka of this Institute were used for most tests, other ascites tumors such as Sarcoma 180, Krebs-2 carcinoma, and the Murphy-Sturm lymphosarcoma were included for comparison. Cellular activity was determined by using a 0.05 per cent methylene blue solution or neotetrazolium chloride as a redox indicator.

In order to make a pour plate, 6 cc. of agar, 1 cc. of rabbit plasma, and 1 cc. of cell suspension in Tyrode's solution were mixed in a test tube and then poured into a Petri dish. Paper disks saturated with test material were placed on the agar surface after it had hardened. Following refrigeration at 4° C. for 10 hours and incubation at 37° C. for 8 hours, the disks were removed and the plates were washed with distilled water and covered with a redox agent. After the excess dye was removed, a circular glass plate was placed on the agar, and the Petri dishes were reincubated for 3 hours at 37° C. The area of unreduced dye, which indicated that the dehydrogenase had been inhibited by the test material and that the tumor cells possibly had been killed, was measured in millimeters. The substances screened by these techniques included many compounds reported in *Cancer Research* Supplement No. 3 (1955), as well as other chemotherapeutic agents and substances of current interest at Roswell Park. Whenever possible the compounds were dissolved in physiological saline and solutions made up of 1000 γ, 100 γ, and 10 γ/ml. Those insoluble in saline were dissolved or suspended in a 0.5 per cent solution of carboxymethyl cellulose in water.

Ascites agar tube. The basic medium for the ascites agar tubes consisted of 0.4 per cent glucose and 1.0 per cent agar in 1/15 M phosphate buffer at *p*H 6.98. Chemicals were prepared at different concentrations as previously described. Ascites tumor cell suspensions of 8×10^6/ml. were made in sterile Tyrode's solution. In each test tube 0.5 ml. of the test chemical solution or suspension and 0.5 ml. of the cell suspension were mixed and incubated for 3 hours in a refrigerator at 4° C. Control tubes contained no test chemical.

Following refrigeration, 2 ml. of medium having 1 ml. of 1:30,000 methylene blue solution for each 100 ml. of medium were added to each test tube. All tubes were incubated at 37° C. The extent of dehydrogenase inhibition was noted following incubation of 3 and 15 hours. Usually there was no difference between the two observations. The results were recorded as follows: + indicates that the dye was not reduced and the tube was completely blue; ±, the tube was either light blue throughout, or only partially reduced at the tip; −, the chemical had no effect and the dye was reduced throughout the tube, except for a thin blue band at the top similar to the control. The experiments carried out with the ascites agar test-tube method paralleled, as far as possible, the ascites agar pour-plate study.

Results

In 1953, a group of investigators agreed to test a series of compounds using 74 systems, including experimental tumors, microbiological systems, differentiation and development, and biochemical synthesis. Twenty-seven compounds were used, including representative compounds of known antitumor activity, agents of biochemical activity in systems other than tumors, a few experimental agents of particular interest at the time, and several samples presumed to be inactive analogues of active carcinostatic agents. It has been possible to obtain 25 of these 27 compounds for testing by the ascites agar plate technique and the ascites agar tube technique. TABLE 1 contains 11 compounds that were found by Creech *et al.* (1955) to be "active" in either the EF or ELD lines of Ehrlich ascites. Seven were found to agree with the *in vivo* results reported, and 4 were found to be completely inactive by both series of tests. It is interesting to note that one of the compounds, 6-mer-

TABLE 1

RESULTS OF ASCITES AGAR TESTS ON COMPOUNDS FOUND ACTIVE BY *IN VIVO* ASCITES TESTS*

Compounds	Agar plates			Agar tubes		
	1000 μg./ml.	100 μg./ml.	10 μg./ml.	1000 μg./ml.	100 μg./ml.	10 μg./ml.
Methyl-bis(β-chloroethyl)amine	40	20	19	+	+	+
A-Methopterin	19	0	0	−	−	−
N-Methylformamide	0	0	0	−	−	−
6-Mercaptopurine	Faint blue	0	0	±	−	−
Urethan	0	0	0	−	−	−
Sodium arsenite†	50	40	15	+	±	±
8-Azaguanine	0	0	0	±	−	−
2,6-Diaminopurine	0	0	0	−	−	−
Diethylstilbestrol (stilbestrol)	22	17	0	±	−	−
2,4-Diamino-5-(4′-chlorophenyl)-6-ethylpyrimidine	9	0	0	±	−	−
Colchicine	0	0	0	−	−	−

* Symbols: +, no dye reduced; ±, dye partially reduced; −, dye completely reduced, except for a thin band at the top similar to that of the control.

† Used in place of potassium arsenite.

TABLE 2

RESULTS OF ASCITES AGAR TESTS ON COMPOUNDS FOUND ACTIVE BY NONASCITES NEOPLASMS

Compounds	Agar plates			Agar tubes		
	1000 μg./ml.	100 μg./ml.	10 μg./ml.	1000 μg./ml.	100 μg./ml.	10 μg./ml.
Hydrocortisone	0	0	0	−	−	−
N-Methylacetamide	0	0	0	−	−	−
Benzimidazole	16*	15	0	+	+	−
1,4-Dimethanesulfonyloxybutane (Myleran, GT 41)	0	0	0	−	−	−
2,4-Diamino-5-(3′,4′-dichlorophenyl)-6-ethylpyrimidine	15	0	0	+	−	−
DL-Ethionine	0	0	0	−	−	−
4-Methoxytoluquinone	40	22	18	+	+	−
Netropsin	0	0	0	−	−	−
Azaserine	Hazy	0	0	±	−	−
1,5-Diaminobiuret	Not available					

* Area of inhibition expressed in millimeters.

captopurine, reported by Mihich *et al.* (1956) as having very little dehydrogenase inhibition activity, gave doubtful results in both the plate and tube test. For comparative purposes, TABLE 2 lists compounds found to be active against only the other experimental neoplasms. Actually, all the compounds listed as having been found active by *in vivo* ascites tests were found to be active by some other tumor system. Of the 10 compounds listed in TABLE 2, only 9 were available; 5 did not respond to either the ascites plate technique or the ascites agar tube technique. FIGURE 1 demonstrates the positive results obtained with 4-methoxytoluquinone on an Ehrlich ascites agar plate. The third and final group (TABLE 3), which was part of the investigation of diverse systems, consisted of compounds that were not considered active with neoplasms. Five of the 6 compounds were available for testing, and no dehydrogenase inhibition was noted with any compound.

Currently, this laboratory has had an interest in a variety of compounds belonging to various chemical groups; the results obtained with several series of these compounds are reported in TABLE 4. FIGURE 2 shows the range of results possible in the agar tube test. The available results obtained with *in vivo* tests using Ehrlich ascites are also presented. Generalization about the correlation between the techniques being discussed and the *in vivo* animal experimentation is difficult, particularly since in some groups very few compounds have been tested.

One possible application of this test can be noted from TABLE 4. Actinomycin D has been reported to inhibit dehydrogenase activity of Ehrlich ascites cells in agar plates at concentrations between 20 and 100 μg./ml. (DiPaolo *et al.*, 1957). No anticell action has been noted with 10 μg./ml. of actinomycin D.

A series of compounds related to actinomycin D has been tested. Of these,

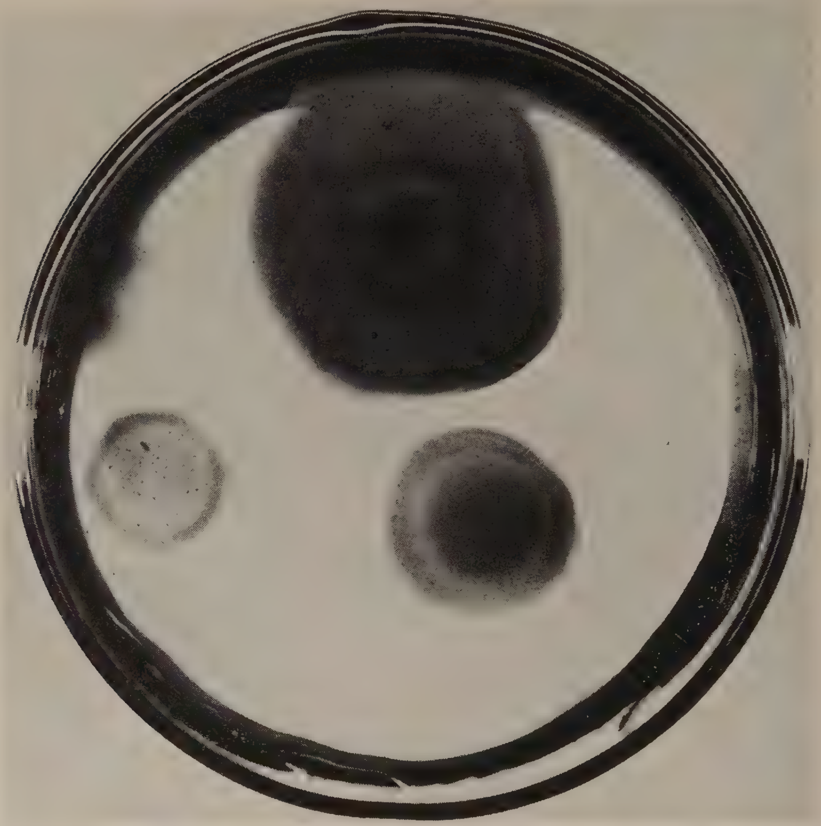

FIGURE 1. Agar pour plate, demonstrating the results obtained with 4-methoxytoluquinone. The dark circles are the areas of unreduced methylene blue; the clear area contains reduced methylene blue.

TABLE 3
RESULTS OF ASCITES AGAR TESTS ON COMPOUNDS INACTIVE BY EXPERIMENTAL NEOPLASMS

Compounds	Agar plates			Agar tubes		
	1000 μg./ml.	100 μg./ml.	10 μg./ml.	1000 μg./ml.	100 μg./ml.	10 μg./ml.
Chloramphenicol (Chloromycetin)	0	0	0	—	—	—
8-Azaxanthine	0	0	0	—	—	—
D-Glucosamine	0	0	0	—	—	—
β-2-Thienylalanine	0	0	0	—	—	—
Deoxypyridoxine	0	0	0	—	—	—
Methylcarbamate	Not available					

TABLE 4

RESULTS OF ASCITES AGAR TESTS WITH A VARIETY OF COMPOUNDS

Compounds	Agar plate			Agar tube			*In vivo* Ehrlich
	1000 µg./ml.	100 µg./ml.	10 µg./ml.	1000 µg./ml.	100 µg./ml.	10 µg./ml.	
Quinones							
2,3-Dichloro-1,4-naphthoquinone	17	14	0	+	+	+	−
Xanthomycin	35	20	15	+	+	+	
Tetracycline	21	15	0	−	−	−	
Surface active							
2-Heptadecyl-2-imidazoline	15	14	0	+	+		
α-Chloro-*N*,*N*-diallylacetamide	25	18	Hazy	+	+	±	
Nitrogen-mustardlike							
$(ClCH_2)_4PCl$	Hazy	Hazy	0	+	±	±	+
Triethylenemelanine	32	23	0	+	+	+	+
Has a 7-C=O group or is potentially capable of having one							
Heptaldehyde	20	18	0	+	±	−	−
Heptaldehyde-bisulphite	0	0	0	−	−	−	−
Reactive double bond							
$HOOC(HC{=}CH)_4COOH$	0	0	0	−	−	−	−
$CH_3O_2C(HC{=}CH)_4CO_2CH_3$				−	−	−	
Purines and pyrimidines							
Caffeine, 8-(pentyloxy)	0	0	0	−	−	−	−
5-Aminoorotic acid	0	0	0	−	−	−	−
Uracil-6-methylsulfone	Hazy	0	0	±	−	−	−
8-Chlorocaffeine	0	0	0	−	−	−	−
8-Ethoxycaffeine	0	0	0	−	−	−	−
8-Allyloxycaffeine	0	0	0	−	−	−	
8-Propoxycaffeine	Hazy	0	0	−	−	−	−
8-Methoxycaffeine	Hazy	0	0	−	−	−	−
6-Mercaptopurine	Hazy	0	0	− to ±	−	−	+
6-Methylmercaptopurine	0	0	0	− to ±	−	−	
2-(3-Pyridyl)-5-NO_2-pyrimidine	0	0	0	−	−	−	−
2-(4-Sulfonamidophenyl)-5-NO_2-pyrimidine	20	0	0	±	−	−	
8-Methylaminocaffeine	0	0	0	−	−	−	−
1,3,7-Trimethyl-9-allyluric acid	0	0	0	−	−	−	−
1,3,7-Trimethyl-9-ethyluric acid	0	0	0	−	−	−	
8-Methylthiocaffeine	0	0	0	−	−	−	
8-Ethylthiocaffeine	0 to hazy	0	0	−	−	−	
Tetramethyluric acid	0	0	0	−	−	−	−
8-Neopentyloxycaffeine	0	0	0	−	−	−	−
8-(2-Methoxyethoxy)caffeine	0	0	0	−	−	−	−
8-(β-1-Pyrrolidylethoxy)caffeine	0	0	0	−	−	−	±
8-(β-4-1-Methylpiperazinyl)ethoxycaffeine	0	0	0	−	−	−	−
8-(β-Bromethoxy)caffeine	0	0	0	−	−	−	−
Aminopterin	19	0	0	+	+	−	−
Planar triangular framework, or could be							
4-(*p*-Dimethylaminostyryl)quinoline	0	0	0	−	−	−	−
1-(*p*-Dimethylaminostyryl)isoquinoline	0	0	0	−	−	−	−
4-(4′-Dimethylaminostyryl)-3-methylquinoline	0	0	0	−	−	−	−
1-(*p*-*N*-Morpholinostyryl)isoquinoline	0	0	0	−	−	−	−
4-(*p*-Dimethylaminostyryl)quinoline reduced	15	0	0	±	±	±	−

Table 4—*Continued*

Compounds	Agar plate			Agar tube			*In vivo* Ehrlich
	1000 μg./ml.	100 μg./ml.	10 μg./ml.	1000 μg./ml.	100 μg./ml.	10 μg./ml.	
3-Aminophenoxazone-2	Hazy	0	0	±	−	−	±
Tetramethylphenoxazone	0	0	0	−	−	−	−
Actinomycin acetate	0	0	0	−	−	−	−
Actinomycin D	−	20	0	+	±	±	±
Actinomycin thio-TEPA	10	0	0	±	−	−	
Actinomycin calcium pantothenate*		0	0		±	−	
Unclassified							
Bacitracin	0	0	0	−	−	−	
Carzinophilin†	24	15	8	+	+	−	++
Erythromycin	Hazy	0	0	−	−	−	
Fumagillin‡	23	13	0	+	+	−	±
Fumagillin alcohol I	Hazy	0	0	±	−	−	±
Fumagillin alcohol Ib	0	0	0	−	−	−	±
Fumagillin alcohol III				+	±	±	
Dihydromonol F ($C_{16}H_{20}O_4$)				−	−	−	±
Gamma globulin§	16	0	0	±	−	−	−
Neomycin	21	15	0	+	+	−	
Penicillin	0	0	0	−	−	−	−
Plasmin¶	19	0	0	±	−	−	−
Polymyxin B	18	0	0	−	−	−	

* 1000 and 100 μg./ml.
† 2500, 250, and 25 units/ml.
‡ 278, 27, and 2.78 μg./ml.
§ 165, 16.5, and 1.6 mg./ml.
¶ 250, 25, and 2.5 units/ml.

only 3-aminophenoxazone-2 has given any indication of inhibition. It is interesting that only this compound has caused slight prolongation of survival time using Ehrlich ascites *in vivo*. Table 4 also includes fumagillin and a group of compounds obtained from fumagillin. Fumagillin itself inhibited dehydrogenase activity in both the plate test and the tube test. Of the 6 compounds related to fumagillin, only 2 showed any activity by the test systems being evaluated. The results obtained with fumagillin alcohol I are of a doubtful nature, judging by both *in vivo* and *in vitro* tests; the results obtained with fumagillin alcohol II have been corroborated with *in vivo* tests; and fumagillin alcohol Ib, which gave negative results with *in vitro* tests, has extended by 50 per cent the survival time of animals bearing Ehrlich ascites.

Previously it was shown that Krebs ascites tumor cells might be substituted for the Ehrlich ascites tumor cells; however, HeLa cells, because of some characteristic of the cells themselves and/or of the medium, were not appropriate for the test as used in this laboratory. Recently, monkey kidney cells obtained from tissue culture were substituted for the Ehrlich ascites cells. Agar plates of a small diameter containing 1 × 10^6/ml., 0.5 ml. of rabbit plasma, and 2 ml. of medium were used. Three compounds, triethylenemelamine, Aminopterin, and mercuric chloride, all of which inhibited dehydrogenase activity of Ehrlich cells, were used in this experiment. None of the plates was satisfactory. This

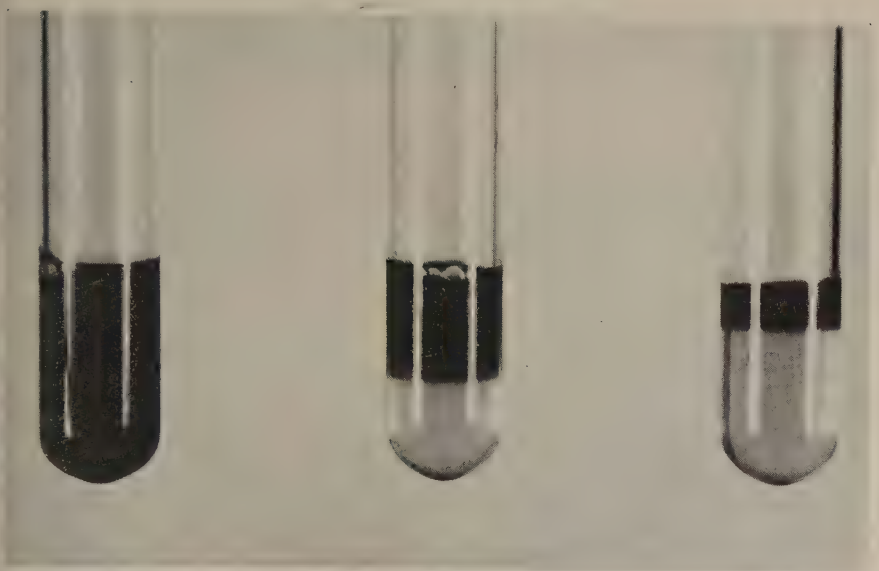

FIGURE 2. A series of test tubes demonstrating the range of results possible using heptaldehyde. The tube on the left shows no dye reduced (+); the center tube shows dye partially reduced (±); and the right tube shows dye completely reduced, except for a thin bane at the top similar to the control (−).

may be due to several factors, including use of too few cells, improper medium, and an incubation temperature unsuitable for these cells.

It has been found that other animal tumor cells such as Sarcoma 180 or the Murphy-Sturm lymphosarcoma may also be substituted for the Ehrlich ascites. A comparison of two mouse ascites tumor-cell suspensions is summarized in TABLE 5. There is very little difference between the two tumor lines. When mouse and rat ascites are compared, as in TABLE 6, it is again noted that the ability of the chemicals to inhibit dehydrogenase activity of the different tumors is essentially the same.

An experiment evaluating the effect of the age of Ehrlich ascites tumors showed that the optimum results were obtained with tumors 7 or 8 days old. For example, if the results obtained with 7-day-old, 10-day-old, and 14-day-old Ehrlich ascites tumors are compared when triethylenemelamine, A-Methopterin, penicillin, and penicillin and mercuric chloride are used, none of the results obtained with the 14-day-old tumor are interpretable, and only some of the results obtained with the 10-day-old tumor are satisfactory when compared to 7-day-old tumors.

An attempt was made to demonstrate the area of inhibition by some indicator of oxidation-reduction level other than methylene blue. A series of indicators reported by Dufrenoy and Pratt (1947) as appropriate in elucidating the mechanisms of action of penicillin on susceptible organisms was used in conjunction with the ascites agar plate test. These indicators were used on a series of plates prepared in the usual fashion with disks containing actino-

TABLE 5
COMPARISON OF EHRLICH-2 AND SARCOMA 180 ASCITES RESULTS IN AGAR TUBE SCREENING TEST

	Ehrlich-2			Sarcoma 180		
	1000 µg./ml.	100 µg./ml.	10 µg./ml.	1000 µg./ml.	100 µg./ml.	10 µg./ml.
Cells only	−			−		
Media only	+			+		
Mercuric chloride	+	+	+	+	+	+
6-Mercaptopurine	±	−	−	−	−	−
Deoxypyridoxine	−	−	−	−	−	−
8-Methoxycaffeine	−	−	−	−	−	−
Neomycin	+	+	−	+	+	+
Bacitracin	−	−	−	−	−	−
Penicillin	−	−	−	−	−	−

TABLE 6
COMPARISON OF MOUSE AND RAT ASCITES IN AGAR TUBE SCREENING TEST

	Ehrlich			Sarcoma 180			Murphy-Sturm lymphosarcoma		
	1000 µg./ml.	100 µg./ml.	10 µg./ml.	1000 µg./ml.	100 µg./ml.	10 µg./ml.	1000 µg./ml.	100 µg./ml.	10 µg./ml.
Cells only	−			−			−		
Media only	+			+			+		
Mercuric chloride	+	+	+	+	+	+	+	+	+
5-Aminoorotic acid	+	−	−	+	−	−	+	−	−
2-Heptadecyl-2-imidazoline	+	±	−	+	+	−	+	+	−
6-Mercaptopurine	−	−	−	−	−	−	−	−	−
8-Methoxycaffeine	−	−		−	−		−	−	
$(ClCH_2)_4PCl$	+	+	±	+	+	−	+	+	−
Deoxypyridoxine	−	−	−	−	−	−	−	−	−
Neomycin	+	+	−	+	+	−	+	+	−

mycin D and polymyxin B, known to inhibit Ehrlich-2 cells, and bacitracin, which had no effect on Ehrlich-2 cells by this technique. TABLE 7 shows that none of the indicators is a satisfactory substitute for methylene blue. This is hardly surprising because, in the agar ascites test, there is no evidence of normal growth, as there is in bacterial agar plate cultures.

Previously, neotetrazolium chloride, which is also an indicator of dehydrogenase activity, was shown to be a possible substitute for methylene blue. The zones of inhibition produced by this dye were sharp but difficult to read because the surrounding area was so pale. Phenol red, a *p*H indicator, was used as an indicator of dead and living cells. In a series of ascites agar tubes containing either triethylenemelamine, mercuric chloride, penicillin, or folic acid, the indicator turned from red to yellow in the tubes containing penicillin or folic acid, indicating production of acid by living cells. No change in color at any concentration was noted with triethylenemelamine and mercuric chloride.

TABLE 7

TEST FOR INHIBITION OF SPECIFIC GROUPS WITH EHRLICH ASCITES AGAR PLATES

Indicator	Group tested for	Expected color reaction		Time after indicator added		
		Inhib. zone	Noninhib. zone	5 min.	3 hrs.	20 hrs.
Cobalt nitrate	Sulfhydryl	Clear	Cloudy	Cloudy	Cloudy	Opaque
Schiff's	Aldehyde	Clear	Red	Pale pink	Pale pink	Pale pink
Chromaffin	Phenol	Orange ring	Colorless	Yellow	Yellow	Yellow
Ferric chloride	Phenol	Clear	Green	Sl. cloudy	Cloudy	Opaque
Copper acetate	Fatty acids	Opaque	Clear	Cloudy	Cloudy	Opaque
Nile blue	Fatty acid	Purple	Blue	Blue	Blue	Blue
Osmic acid	Polyphenols	Dark ring	Clear	Clear	Sl. brown	Black
Millon's reagent	Tyrosine	Red ring	Clear	Sl. cloudy	Cloudy	Opaque
Toluidine blue	RNA	Lavender	Dark purple	Blue	Blue	Blue
Methyl green	Dehydrogenase	Green	Pink	Green	Green	Green

TABLE 8

CHANGES IN REDUCTION TIME OF DIFFERENT UNTREATED CELL CONCENTRATIONS IN TUBES

Cells × 10^6/tube	5	4	3	2	1	0.5
Reduction time in hours	.75	1	1.5	2	2.5	3
24 Hours later	Reduced	Reduced	Reduced	Unreduced	Unreduced	Unreduced

This is in agreement with the results obtained when methylene blue is used as an indicator.

Since such compounds as nitrogen mustard and triethylenemelamine responded by these tests, an experiment was attempted using X radiation in place of a chemical. A series of ascites agar plates was exposed to 5,000, 10,000 and 20,000 r. Methylene blue was reduced in all of the plates. When X radiation of 12,000, 24,000, 48,000, and 96,000 r was applied to a series of ascites agar tubes, reduction took place in all tubes, indicating that dehydrogenase activity was not inhibited. Dye in control tubes containing ascites cells was reduced at 2 hours, while contents of tubes with medium only showed no reduction.

As a routine, the agar tube test was evaluated after 3 hours and overnight incubation. Ordinarily there was no change in the results. The reduction time by different concentrations of cells in untreated agar-ascites tubes containing Ehrlich cells is indicated in TABLE 8. These results were obtained in an experiment in which cells were not previously refrigerated. On tests carried out as indicated in the description of materials and methods it was found that 4 × 10^6 cells/tube gave results that permitted accurate evaluation of the compounds.

Summary

In summary, the techniques described are of value, especially when used in conjunction with some experimental animal technique. They present an opportunity for empirical screening of compounds, as well as for studying the relationship between structure and activity as a basis for further synthetic work for cancer chemotherapy.

Acknowledgments

We acknowledge the generous assistance of C. T. Bahner, J. H. Burckhalter, E. W. Malmberg, and D. S. Tarbell, and thank several pharmaceutical companies, including Abbott Laboratories, North Chicago, Ill., Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., and Merck & Co., Inc., Philadelphia, Pa., for furnishing materials used in these experiments.

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Discussion of the Paper

Question: What was the purpose and method of refrigeration at the beginning?

DiPaolo: Refrigeration is used to allow a longer period of contact with the chemicals. In the plate test the complete plate is made and refrigerated first. In the tube test a cell suspension containing chemicals for a period of time is refrigerated. Following this, the agar and the indicator are added and the suspension is then incubated at 37° C.

Comment: We tried this test about a year ago and achieved no good correlation with animal tests. In fact, streptomycin and hydroquinone were our most active materials in the test-tube system. We tested thirty different actinomyces filtrates and obtained no correlation whatsoever with the activity in animal systems.

DiPaolo: All I can say is that we found actinomycin D to be positive in the system we used, and we have been interested in compounds that might be related to actinomycin. We found that screening of 3-aminophenoxazone-2 produces a slight zone, which agrees with our animal tumor system.

ROLE OF CHANCE OBSERVATIONS IN CHEMOTHERAPY: *VINCA ROSEA*[*]

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This paper may seem somewhat unorthodox in a monograph on screening procedures. However, we are eager to present the role of chance in obtaining new leads for chemotherapy and to illustrate this with a specific example. The results of our research, which are presented here in detail for the first time,[1, 2] should not be considered in terms of a new chemotherapeutic agent, but rather in terms of a chance observation that has led to the isolation of a substance with potential chemotherapeutic possibilities.

The cancer worker in the smaller institution or the academic department must view with awe the vast chemotherapeutic screening projects in progress in the United States; at the same time, however, he must consider what contribution he is in a position to make. Perhaps the role of chance observation is neglected in his consideration of ways of searching for new agents. Although somewhat irregular in comparison with the systematic prediction, synthesis, and screening of an entirely new series of compounds, chance observations may well be worthy of greater consideration than they have received.

The chain of events that led to our present problem originated in our laboratory in 1949 because we had been working with, and were somewhat intrigued by, various plant extracts to which historical hearsay had ascribed empirical uses by primitive peoples. The disease of cancer was certainly far from our thoughts when we learned of a tea made from the leaves of a West Indian shrub that was supposedly useful in the control of diabetes mellitus. C. D. Johnston of Black River, Jamaica, Federated West Indies, had been curious about the reputed benefits of a tea made from the leaves of the white-flowered periwinkle, *Vinca rosea*; he forwarded a supply of the material to us. The effects of extracts of the plant given orally to experimental animals proved disappointing, and essentially no alterations in the blood sugar of normal or diabetic rats or rabbits could be induced. Similarly, no effect on the response to glucagon was found. Whether there is any basis for the use of this material in diabetes could not be ascertained, despite a brief visit by J. C. Rathbun and H. A. McAlpine of our department to Johnston. As we became aware later, a number of earlier workers had investigated the properties of periwinkle extracts on carbohydrate metabolism, with essentially negative results,[3-5] although a proprietary preparation, Vinculin, had been marketed in England for some time as a "treatment" for diabetes. More recently Hugh-Jones[6] has com-

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mented that although periwinkle extracts may perhaps reduce the glucosuria in diabetics, at the same time there is an associated increase in blood sugar.

With the intention of increasing the possible effectiveness of water extracts of periwinkle, we administered these by injection to rats. In contrast to the oral use of such material, the animals were obviously affected, but in an unexpected manner. In most cases death followed a single injection in five to seven days, and the rats at autopsy showed multiple abscesses, particularly in the liver and kidneys, the obvious result of a septicemia. On culture, *Pseudomonas* spp. were consistently found, according to a private communication from R. G. E. Murray of the Department of Bacteriology, University of Western Ontario. Since these organisms were not found in the extract, it was apparent that an endogenous infection had occurred in the treated animals. Curiously enough, at the time of these experiments the same organisms were isolated from rats being used in other studies in the laboratory that were receiving large doses of cortisone and bore abscesses. Apparently some natural barrier to infection was being depressed by both types of treatment. Further studies readily localized the action of the *V. rosea* extracts—a rapidly falling white blood count, granulocytopenia, and profoundly depressed bone marrow were always associated with it. This unusual finding was consistently produced in the rat, even after a single injection, and the changes appeared to be far more severe than those ascribed to the commoner drugs causing agranulocytosis. Consequently, it was made a subject of further study. From the observation that a water extract of 2 gm. of the dried leaves of periwinkle caused a reduction in the white blood count to below 10,000 in 3 to 5 days, as well as a virtual disappearance of neutrophil leukocytes, it was decided to attempt some degree of chemical purification. Although a number of workers in the laboratory were able to work spasmodically on this problem, an intensive chemical study was not started until 1955.

One early problem was to determine the activity of the various species of *Vinca* and the location of the active substance in the plant. Although previous studies have been made on *V. major* and *V. minor*, and various substances have been isolated,[7-11] extracts of these species did not affect the blood picture in the rat in our studies. Other investigators interested in chemical studies with *V. rosea* have not, to our knowledge, reported any antihemopoietic action.[12, 13] Extracts of both the white and pink varieties of *V. rosea* were found to be highly active. The roots, stems, and leaves all contained active material but no detectable activity was found in the seeds. The leaves, which could most readily be extracted, contained the highest proportion of activity. Cultivation near London of *V. rosea* was undertaken by H. Swain and Sons. The plant was grown as an annual, and activity was determined at different periods of growth. Although active extracts could be obtained, the yield was about one fourth of that found in material obtained from the West Indies, where the plant grows wild and is a perennial.

Using the depression of blood count of the normal rat as a measure of assay, it was soon found that using acid extraction of the dried plant was an advantageous procedure, and that a major portion of activity was precipitated when the extract was basified. The precipitated activity was soluble in chloroform, benzene, and aliphatic alcohols. These observations indicated that the active principle was probably an organic base. In addition, the crude extracts gave typical alkaloid reactions with color and precipitating reagents such as Dragendorff's and Mayer's, silicotungstic acid, and picric acid.

In determining methods for isolating the active principle or principles, it seemed desirable to link each step of a chosen procedure to a biological assay and establish as nearly as possible a "balance sheet" of the distribution and recovery of activity after chemical manipulation. It was hoped thereby not only to determine the efficiency of a particular fractionation procedure, but also to reveal either the presence of additional active principles or of synergism among various components of the original fraction.

The activity could be recognized only by the ability of this material to produce a peripheral granulocytopenia and depression of the bone marrow. Assay consisted of daily determination of the white cell count in blood taken from the tail and intermittent examination of bone marrow. Titration of doses of the active material showed a sharp delineation between an effective and noneffective dose. Within the nonlethal range of activity there was some correlation between the size of dose and the degree of response obtained. The use of alterations in the blood count of the normal rat as an assay procedure may have been unwise, and a test method against a transplantable leukemia might have proved more exacting. Initially, however, we were not thinking in terms of cancer chemotherapy; moreover, the adopted assay could be carried out in a few days and required only small amounts of extract.

It was soon found that ethanol containing acetic acid (10 per cent by volume) extracted the activity very efficiently from the dried leaves of *V. rosea*; this solvent mixture has been used in all recent work for preparing the initial crude extract. Since it seemed highly probable that this extract was a complex mixture of organic bases with differing *p*K values, the distribution of the active principle among organic solvents and aqueous media at various *p*H values was examined. From the data obtained, the separation scheme illustrated in FIGURE 1 was devised for the preliminary fractionation. The original aqueous solution was extracted with benzene or chloroform at *p*H 4, 7, and 8.5. The biological activity of the extracts thus obtained in a typical experiment using *V. rosea* of Jamaican origin is also shown in FIGURE 1. It may be seen that, of the 3 extracts obtained, the one from benzene at *p*H 7 contained the greater part of the activity—8 to 10 times that in the other fractions—although it weighed less than one third of the total material extracted.

The material extracted at *p*H 7 with benzene has given uniform and consistent responses in 3 strains of rats, 2 strains of mice, and in the guinea pig. The "standard response" shown in FIGURE 2 was obtained when rats were given single I. P. injections of 7.0 to 7.5 mg./kg. of the material dissolved in 0.3 ml. of propylene glycol. Lethal doses contained 3 to 4 times this amount of material.

Twenty-four hours after injection of the active material, the peripheral blood showed a moderate neutrophilia. This was a nonspecific reaction that could

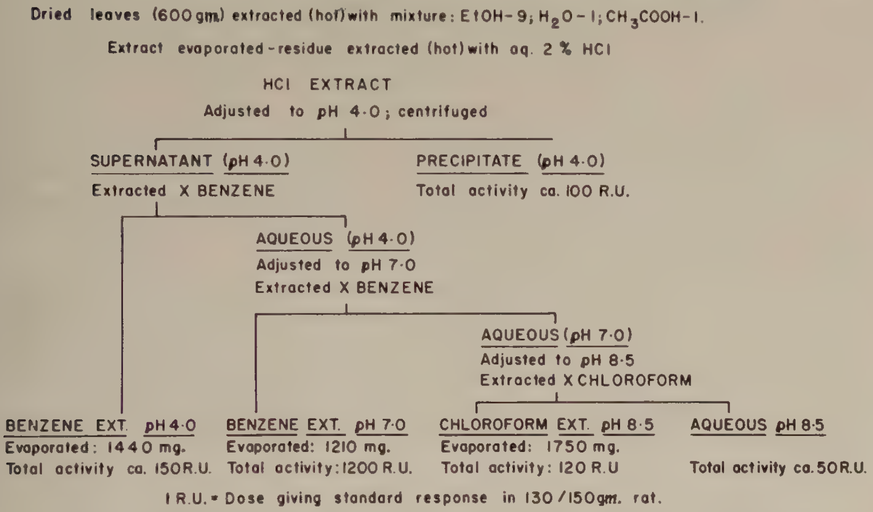
Dried leaves (600 gm) extracted (hot) with mixture: EtOH-9; H_2O-1; CH_3COOH-1.
Extract evaporated-residue extracted (hot) with aq. 2 % HCl
HCl EXTRACT
Adjusted to pH 4·0; centrifuged
SUPERNATANT (pH 4·0)
Extracted X BENZENE
PRECIPITATE (pH 4·0)
Total activity ca. 100 R.U.
AQUEOUS (pH 4·0)
Adjusted to pH 7·0
Extracted X BENZENE
AQUEOUS (pH 7·0)
Adjusted to pH 8·5
Extracted X CHLOROFORM
BENZENE EXT. pH 4·0
Evaporated: 1440 mg.
Total activity ca. 150 R.U.
BENZENE EXT. pH 7·0
Evaporated: 1210 mg.
Total activity: 1200 R.U.
CHLOROFORM EXT. pH 8·5
Evaporated: 1750 mg.
Total activity: 120 R.U
AQUEOUS pH 8·5
Total activity ca. 50 R.U.
1 R.U. = Dose giving standard response in 130/150 gm. rat.

FIGURE 1. Preliminary fractionation of crude *Vinca rosea* extract. Each aqueous portion was extracted 3 times with an equal volume of solvent, and the combined solvent backwashed twice with one fortieth vol. water. All solvent extracts were taken to dryness *in vacuo* at 55° C. in a rotary evaporator.

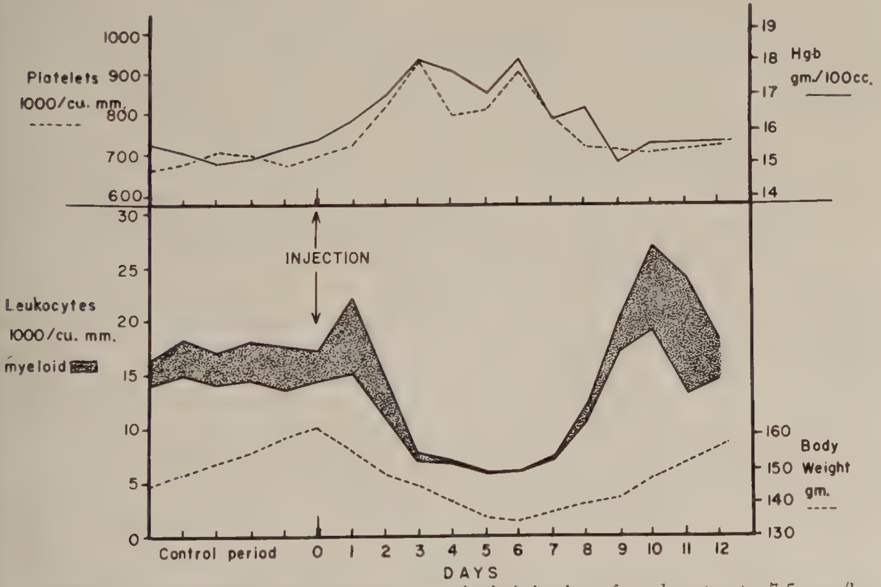

FIGURE 2. Peripheral blood response to a single injection of crude extract—7.5 mg./kg. material extracted by benzene at *p*H 7.0. The total height of the curve is equal to the total leukocyte count.

be elicited by the introduction of any irritant material. Thereafter a rapid fall in total white count developed, accompanied by a decrease of both myeloid and lymphoid elements but most pronounced in the former. A severe peripheral granulocytopenia was apparent by 48 hours, and this progressed for another 4 to 5 days. The leukopenic phase terminated abruptly in a leukocytosis that varied in degree, but generally exceeded 30,000 leukocytes/cu.mm. Regardless of the height reached by the leukocyte count, the period of leukocytosis was characterized by an excess of myeloid cells. Lymphoid elements rarely exceeded their normal values. The return to normal was rapid and unremarkable.

The leukopenic phase was accompanied by increased values for red cells, packed cell volume, and platelets. These reached their highest values by the fourth or fifth day, which was coincidental with the leukocytes reaching their lowest values. These values gradually returned to normal.

Weight loss was found consistently and was evident as early as 24 hours after injection; it persisted throughout the course of the response. Other toxic symptoms included a severe diarrhea, which appeared about 48 hours after injection and lasted for 8 to 12 hours. In fatally intoxicated animals the diarrhea was present until death. A sharply restricted food and water intake was also noted.

The first discernible marrow lesions appeared by 24 hours and consisted of a shift of cell population toward the immature or blast cell, with little or no alteration in the total number of cells. Thereafter the marrow rapidly became depleted, so that by 72 hours the femoral marrow had been converted to a thin, purple fluid. Smears showed a markedly depleted marrow containing some nucleated red cells, cells that correspond to reticulum cells, and mature lymphocytes. Megakaryocytes were not affected early. In fatally intoxicated animals the marrow showed only a few mature lymphocytes, an occasional denuded megakaryocyte nucleus, and a rare blast cell.

Histological sections of the gut showed dilation of vessels of the mucosa and submucosa; the crypts were filled with leukocytes, but there was little desquamation of the epithelium. There was no evidence of damage to other abdominal viscera, and there appeared to be little effect on lymphoid tissue.

Since attempts to isolate an active crystalline compound or derivative from the material extracted with benzene at *p*H 7 were unsuccessful, further fractionation was undertaken. Chromatographic separation on alumina gave early promise of partial resolution of the active principle. However, numerous trials with aluminas of various activities and with a wide range of solvent mixtures changed stepwise in composition did not lead to an entirely satisfactory separation. Analysis by a paper chromatographic method revealed that there was considerable overlapping of substances that appeared to be closely similar in the effluent from the column. The method of "gradient elution" whereby the composition of an eluting solvent mixture is changed gradually rather than stepwise has given greatly improved resolution in certain instances, for example, steroid separations.[14, 15] The method has not yet been employed to any extent in alkaloid separations. However, in the present work it gave a significantly better separation of the active principle from adjacent inactive substances, especially if the benzene extract was washed with dilute alkali to remove phenolic and tarry materials prior to chromatography. No loss of biological activity resulted from this operation.

FIGURE 3 shows the separation obtained when this washed and dried extract was placed on an alumina column—Woehlm "almost neutral" Grade IV–V—and eluted with a solvent mixture whose composition changed linearly from benzene 65 per cent v/v; methylene chloride 35 per cent v/v at the start to pure methylene chloride at the end of fraction 18. Each fraction from this column was assayed. In those cases where little or no activity was found, the dose was increased up to a maximum of one thirtieth of the total fraction. As shown in FIGURE 3, this system gave good resolution of the activity, which was now found to be present primarily in only a small weight of material relative to that applied to the column. Thus, the fraction with maximum activity (FIGURE 3, fraction 9) weighed only 27.5 mg. in contrast to the 1380 mg. applied. The fraction was semigranular and pale brown.

The results of the bioassay showed a tenfold purification. Standard hematological responses were obtained with doses containing 0.70 to 0.80 mg./kg. of material. Doses in excess of 3 to 4 times this amount were lethal. In general, the standard response was the same as that produced by the cruder fraction, but there were some differences. Injection of a minimum effective dose rarely elicited the granulocytosis at 24 hours, and the marked granulocytosis during recovery was no longer apparent. Diarrhea did not constitute a prominent part of the response, as it had with the crude fraction. When it did occur, it was transient, lasting only 3 to 4 hours, was unaccompanied by evidence of gut lesions, and was elicited only at high dose levels. Weight loss was still considerable. The duration of peripheral response was the same as for the crude extract, but there seemed to be a greater sparing action in ymphoid tissue.

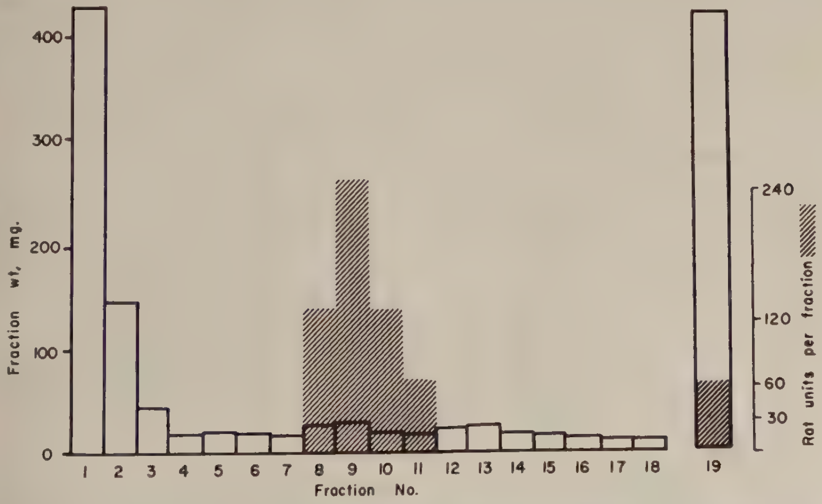

FIGURE 3. Gradient elution chromatography of alkali-washed pH 7.0 benzene extract from 600 gm. dried leaves. Material applied: 1380 mg.; recovered, 1330 mg. Absorbent: alumina, Woehlm "almost neutral" Grade IV–V, 120 gm. Fractions 1–18, 2700 ml. benzene 65 per cent v/v, methylene chloride 35 per cent v/v diluted progressively with 2500 ml. methylene chloride; gradient was linear. Fraction 19 was eluted with 600 ml. ethanol.

At this point, reversals of the peripheral and marrow effects were attempted, using folic and folinic acid, thymine, cytidine, guanine, and vitamin B_{12}. None of these agents showed any alleviation of the hematologic symptoms, nor did they in any way affect the course of intoxication.

FIGURE 3 shows that activity has also been found in fraction 19. This fraction, which was eluted with ethanol from the column, was a complex, dark-brown tarry mixture; paper chromatography showed that it contained a number of additional alkaloids.

Assay of fraction 19 revealed that it, too, produced a standard hematological response, but only at doses containing 7 to 8 times the weight of material active in fraction 9. At doses containing less than 5 to 6 mg./kg. no effects were elicited. The hematological response was accompanied at all effective dose levels by severe diarrhea, weight loss, and gut lesions—in other words, it was identical with that observed after administration of the *p*H 7.0 fraction prior to chromatography.

Further biological investigation revealed other characteristics between the material extracted at *p*H 7.0 and fraction 19, which served to differentiate the main active region, fraction 9, from fraction 19. Both the unchromatographed material and fraction 19 were able to suppress the uterine response to stilbestrol; this activity could be abolished by either the prior administration of folic acid or the concurrent administration of folinic acid. Such activity was not associated with fraction 9.

Standard doses of the active material extracted at *p*H 7.0 and of fraction 19 were administered daily for 3 days to separate groups of immature female rats. On the second and third days the animals also received 0.60 mg./kg. of stilbestrol. All injections were made subcutaneously. FIGURE 4 shows

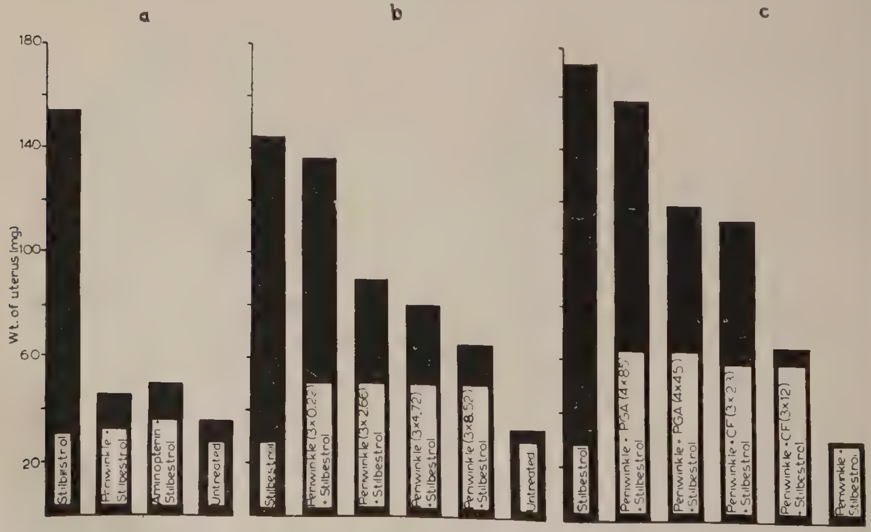

FIGURE 4. Uterine response to stilbestrol: (a) after *V. rosea* extract, 8 mg./kg. × 3; (b) dose titration of *p*H 7.0 fraction; and (c) reversal of inhibition by folic and folinic acid.

TABLE 1
UTERINE DATA FOR FRACTION 19

Number of rats	Material	Dose/kg.	Uterine wt.	Body weight	
				Initial	Final
6	Fraction 19 Stilbestrol	3 × 5.6 mg. 2 × 0.6 mg.	36 ± 1.3	46 ± 1.3	43 ± 0.8
6	Fraction 19 Stilbestrol	3 × 3.0 mg. 2 × 0.6 mg.	88 ± 3.6	60 ± 4.8	67 ± 3.1
6	Fraction 19 Stilbestrol	3 × 1.5 mg. 2 × 0.6 mg.	106 ± 3.7	59 ± 3.6	66 ± 4.2
6	Stilbestrol only	2 × 0.6 mg.	149 ± 3.8	61 ± 3.8	69 ± 7.0
6	Nil		38 ± 4.1	60 ± 4.1	66 ± 6.3
6	PGA PW Stilbestrol	4 × 90.0 mg. 3 × 5.9 mg. 2 × 0.6 mg.	153 ± 11.9	59 ± 1.3	64 ± 4.6

the results obtained using the unchromatographed extract. Titration of the dose of this material showed a distinct relationship between the dose and the uterine response to stimulation by a standard dose of stilbestrol (FIGURE 4*b*). The reversal of this inhibition by folic and folinic acid is shown in FIGURE 4*c*.

Similar results were obtained with fraction 19 of the chromatographed material (TABLE 1). Reversal studies, however, revealed an additional feature that was not found with the material prior to chromatography. Reversal of the uterine depressant action of fraction 19 by folic or folinic acid was accompanied by prevention of the hematological response, whereas at no dose level of any metabolite was it possible to prevent the blood and marrow response to effective doses of the unchromatographed extract.

The fraction with maximum activity (FIGURE 3, fraction 9) has been shown by paper chromatographic analysis to contain at least seven substances, as revealed by inspection under ultraviolet light, and by treating the paper with Dragendorff's reagent. When the material in the areas corresponding to the visualized spots in the paper was eluted and assayed in rats, activity was found in one "spot" only. The activity recovered in this single spot approximated that applied to the paper. This "active spot" gave a pronounced color with Dragendorff's reagent and was apparently a major component of the material in the fraction.

As yet, only limited amounts of the material in fraction 9 have been available, and thus far attempts to crystallize the material have not been successful. However, a well-defined crystalline derivative, the sulfate, has been prepared from this fraction. This derivative crystallized from ethanol as a mass of minute, colorless feathery needles illustrated in the photomicrograph shown in FIGURE 5.

The crystalline compound was readily soluble in water and methanol, and sparingly soluble in ethanol. Its solution in water gave an immediate pre-

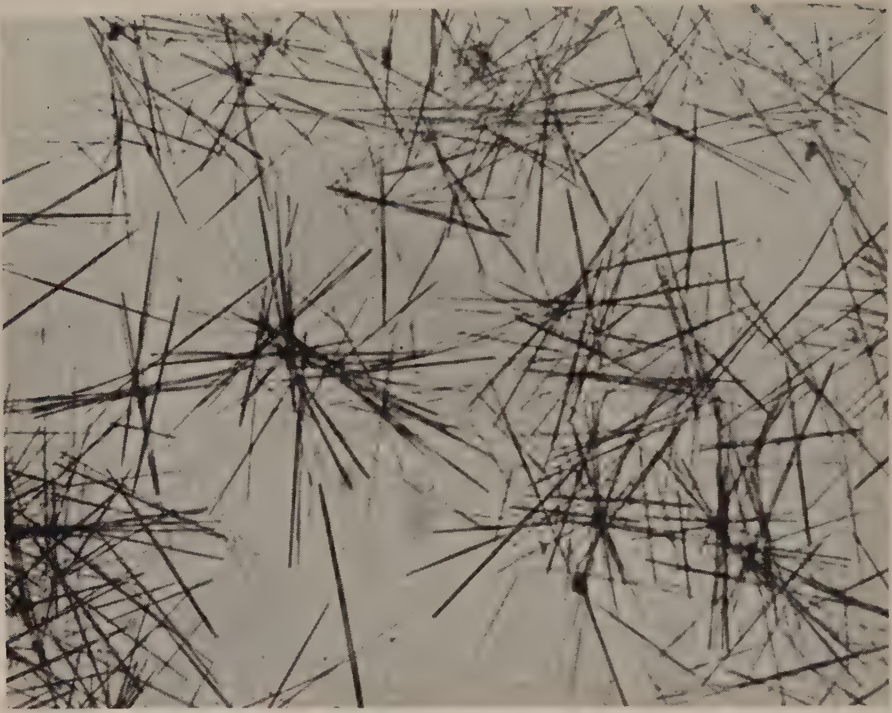

FIGURE 5. Active crystalline sulfate (× 180). Nine mg. of fraction 9 (FIGURE 3) was suspended in 2 ml. water and acidified with dilute H_2SO_4 to *p*H 3.75. The solution was filtered and allowed to evaporate *in vacuo* at room temperature over concentrated H_2SO_4. Residue was dissolved in 0.5 ml. methanol and diluted with 6 ml. hot ethanol. The crystals appearing on cooling were recrystallized from hot ethanol.

cipitate with picric acid and with Dragendorff's reagent. A preliminary analysis of a sample recrystallized from ethanol and dried at 65° C. *in vacuo* indicated the empirical formula: $C_{24}H_{35}N_2O_7 \cdot \frac{1}{2}(H_2SO_4)$. Calculated: C, 56.25; H, 7.1; N, 5.5; S, 3.1. Found: C, 56.65; H, 7.2; N, 5.5; S, 2.9. Heated in an evacuated capillary, the sulfate began to soften and darken at 245° C. and melted with decomposition at 285 to 290° C.; $[\alpha]_D^{22}$ $-36.9°$ (c. 1.01 in methanol). $\lambda_{\text{Max.}}^{\text{MeOH}}$ 262 and 289 mμ (log ϵ 3.69 and 3.58, assuming mol. wt. 512.5), shoulder at λ 297 (log ϵ 3.55).

The free base was obtained by neutralizing an aqueous solution containing a few milligrams of the sulfate. It was extracted with methylene chloride, and the residue left after evaporating the solvent was dissolved in boiling cyclohexane. The free base separated on cooling as a pure white amorphous powder, m.p. 192 to 195° C., becoming glassy at 165° C.; $[\alpha]_D^{23}$ $-32.2°$ (c. 0.88 in methanol). $\lambda_{\text{Max.}}^{\text{MeOH}}$ 262 and 288.5 mμ, shoulder at λ 296.5. The ultraviolet absorption curve was almost identical with that of the sulfate. Thus far this compound has been isolated in small amounts only, and has not yet been obtained in crystalline form.

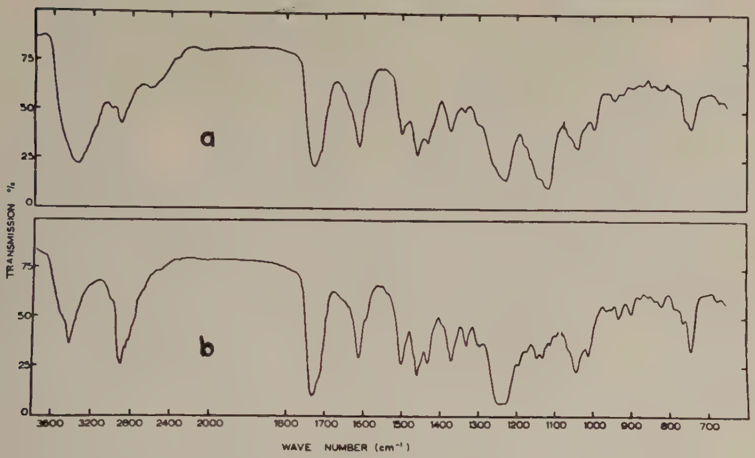

FIGURE 6. Infrared spectra of biologically active substance isolated from *V. rosea*: (a) crystalline sulfate and (b) free compound. Both samples were run in KBr disks on a Perkin-Elmer model 21 instrument.

The infrared spectra (FIGURE 6) of the sulfate and free base (KBr) were very similar; for example, both showed pronounced absorption at approximately 2.9 μ (NH-absorbing region), 5.8 and 8.1 μ (carbonyl and ester regions), approximately 13.4 μ (di-substituted benzene ring absorption?), and 3.4 and 6.8 to 7.3 μ (aliphatic structures?).

The present evidence is not sufficient to establish the class of alkaloid to which the active substances belong. However, the presence of reserpine and alkaloids of allied structure in plants of the *Vinca* genus, including *V. rosea*,[13] is of some interest, since the alkaloids of a particular plant are frequently all related by a common structural feature. For purposes of identification, the name Vincaleukoblastine is suggested for the crystalline substance described.

An injection of the free base corresponding to 0.35 mg./kg. of the crystalline sulfate elicited the typical peripheral response shown in FIGURE 7. No changes in the peripheral blood were observed until 36 to 48 hours after injection. Thereafter, a severe granulocytopenia developed and was maintained for 4 to 5 days. Lymphocytes were only slightly reduced. Recovery commenced on the fifth or sixth day after injection, and the leukocyte count and myeloid cells gradually returned to normal over a period of 2 to 3 days. There was no striking initial granulocytosis like that observed with the crude fraction.

The toxic symptoms accompanying the injection of 0.35 mg./kg. were minor. There was a slight weight loss for 24 hours after the injection, but thereafter the animal gained weight at a normal rate. The response was unaccompanied by diarrhea or gut lesions, even after administration of lethal doses of the substance.

By 18 to 24 hours, a shift in the population of marrow cells could be observed. At first this consisted of an increase in immature cells, but it was later followed by a rapid and severe depletion of all marrow cells. By 72 hours,

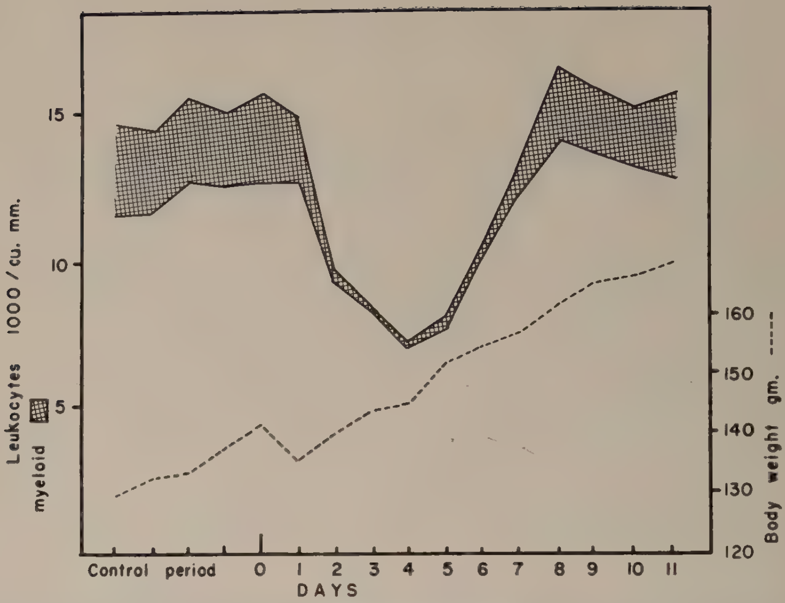

FIGURE 7. Peripheral response to a single intraperitoneal injection of 0.35 mg./kg. of the free base liberated from the crystalline derivative. The total height of the curve is equal to the total leukocyte count.

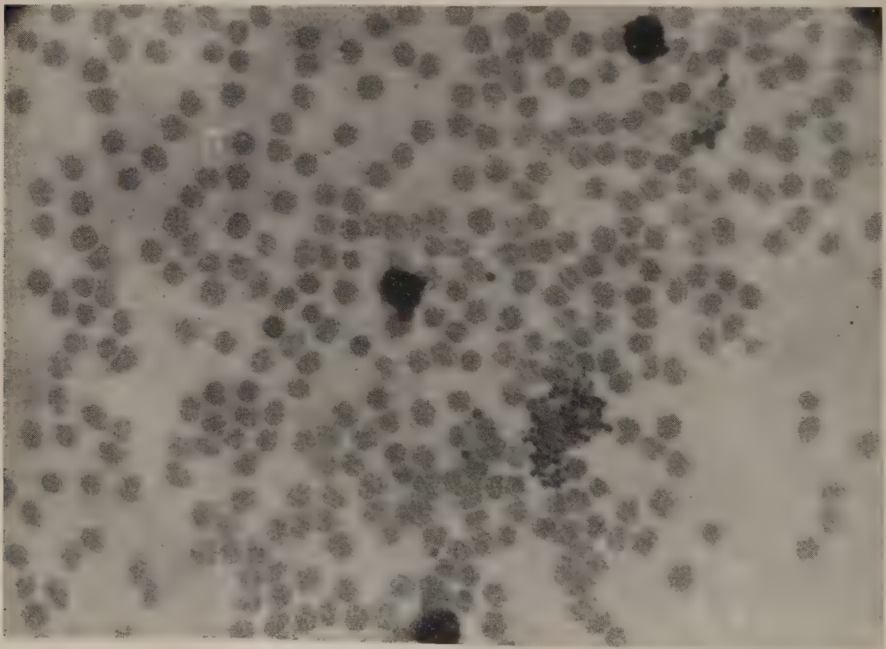

FIGURE 8. Femoral bone marrow of rat treated with free base from crystalline material. × 450.

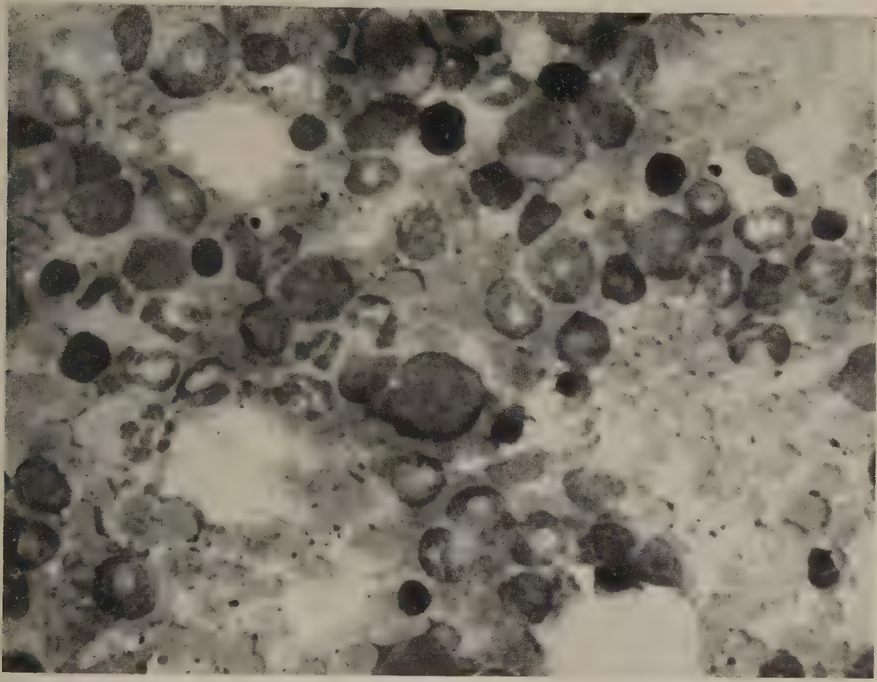

FIGURE 9. Femoral bone marrow of the normal rat. × 650.

even at nonlethal doses, the marrow consisted of a thin fluid containing a scattering of mature lymphocytes. Marrow cells such as myelocytes and metamyelocytes were absent, and blast cells and other immature forms could be found only with difficulty. FIGURE 8 shows such a marrow, which may be compared with the normal marrow shown in FIGURE 9.

Limited investigations for carcinostatic activity have been made from time to time, as material was available. The crude material possessed some definite carcinostatic activity against transplantable mammary adenocarcinoma in DBA/JAX mice and against a transplantable sarcoma in the rat. Further screening of the crystalline material is in progress and will be reported later.

Acknowledgments

The authors are indebted to J. B. Collip for his interest in this problem, to K. K. Carroll, Helena Robinson, Shirley Barrett, E. Pedersen, and A. Andersen, all of this laboratory, who assisted in the research; to Marilyn Dynes, Bette Thomas, and G. Carpenter for technical assistance; and to E. B. White, Science Service Laboratory, London, Ontario, for running the infrared spectra and for interpreting the curves shown in FIGURE 6.

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Part VIII. Considerations in Experimental Cancer Chemotherapy

INTRODUCTION TO PART VIII

By John T. Litchfield, Jr.
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I think we can recognize a thread running through this monograph, that it is a review of the older techniques and, especially, a presentation of newer techniques that ultimately may be used for screening and evaluating antineoplastic agents. One of these newer techniques may replace, in time, the techniques upon which we rely today.

At present the use of the transplanted or inoculated tumor in rats or mice is still our basic tool for screening. I think all of us admit the defects of this technique; nevertheless, we cannot discard it until a better one is found.

In recent years we have found ways of using this tool more efficiently by taking advantage of developments in the field of statistics. In doing this I think we have recognized fully the role played by Parkinson's Law, namely, that work expands to fill the time available whether the work is of consequence or not. In fact, we have actually taken advantage of this law. Screening facilities were available and were increasing in size; therefore, many substances would be screened in any case in accordance with this law, because every screening facility always immediately fills itself to capacity by replacing procedures previously used. What we have done is to learn how to make the facility handle two to four times as great a load by using these newer techniques.

STATISTICAL PROBLEMS IN A MASS SCREENING PROGRAM

By Peter Armitage* and Marvin A. Schneiderman
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Introduction

By "mass screening program" we mean a procedure for examining a large series of materials in such a way that the minority are retained for further investigation while the majority are rejected or set aside. Different stages of the screen may involve different types of procedure. In cancer screens, for example, we may be concerned with tests on experimental animals, with other biological laboratory tests, with human investigations, or with combinations of these approaches.

In examining particular problems we find that the optimal strategy usually depends on the nature of the materials entering the screen. This is not only unknown, but may be difficult to estimate with even slight accuracy until the program is well under way. Furthermore, the type of material submitted may well change as the program unfolds, since any positive findings will stimulate interest in related chemical compounds.

The optimal strategy for any stage of a screening program depends also on the precise formulation of aims. In general, we wish to increase the relative frequency of effective materials, but this vague desire can be rendered more precise in various ways. For example, we may wish to retain, with high probability, all materials with a biological activity beyond a certain level; or to retain a fixed proportion of all materials submitted; or to retain a fixed number each year, in order to meet exactly the capacity of the workers to whom the accepted materials are passed. Each of these aims will determine different types of optimal scheme.

Of the screen as a whole, we should require not only that the proportion of effective materials be increased progressively with the several stages of screening, but also that it be increased to a reasonably high value. An increase of this proportion from 1 in 100,000 of entering materials, for instance, to 1 in 50 after several stages, would indicate that still more screening was necessary.

Animal Screening

Choice of system or systems. If we assume for the moment that all systems are equally convenient and equally expensive, our ideal choice would be the one that gives the best prediction of materials effective in humans. Unfortunately, the performance in humans of most of the materials is largely unknown. A minimal requirement is that the animal screen should have a very small chance of rejecting any material known to be effective in man. The Gellhorn-Hirschberg report[1] compared the sensitivity of various systems to certain compounds then advocated as useful in the treatment of malignant disease. These compounds were relatively few in number and, in view of the lack of controlled clinical trials, some may have been of doubtful value. As part of the regular Cancer Chemotherapy National Service Center (CCNSC) documentation program, our colleagues E. W. Foltz and A. L. Hobbs have gathered reports of materials said to be of use against human cancer. These reports still require critical assessment, after which their performance in our animal screen will be examined.

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The three tumor systems in use at CCNSC—Ca 755, S 180, and leukemia L 1210—were chosen on the basis of the Gellhorn-Hirschberg report,[1] but at a later time it may prove necessary to use other systems, such as human tumors, tissue culture, or microbiological systems. The choice of a system should be under continuous review, for the information yielded by any single set of systems will gradually suggest the need for modifications or radical changes.

Strategic considerations. Abraham Goldin and Nathan Mantel of the National Cancer Institute, in an unpublished manuscript, have pointed out the merits of using acceptance criteria of varying stages of severity. These may be within the same system, for example, the occurrence of deaths at various times after treatment, or they may be on different systems. If all tests are equally expensive, it may be best to use only the most severe criterion, that is, one that tends to concentrate the most effective materials among a very small number of compounds passing the test. If such a test is expensive, it may be preferable to start with a cheapter test although its concentrating power is low, and to reserve the more severe test for later use. Similarly, one could plan a series of tests of increasing severity.

Another strategic problem is that of how to deal with toxicity. One possibility is to run a full-scale toxicity test for each compound, and then to evaluate antitumor effect at the maximum tolerated dose (however, the greatest therapeutic effect may occur at less than the maximum tolerated dose). Another approach, and the one we follow at CCNSC, is to screen the compound without delay at the highest possible dose, and then retest at a lower dose if the first proves toxic. Occasionally, more than one adjustment may be necessary. The dilution factor used for the adjusted dose is based on some preliminary investigations of the relationship between toxicity and dose. On the average, each compound is tested at about 4.5 doses for the total trials with the 3 tumor systems, which suggests that the method is economical.

Design of experiments. When the system and the test criteria have been chosen, we are faced with what might be called tactical problems in the design of the screening experiments.

In general, the natural course of an experimental tumor system is so variable that a control group of animals treated with an inert material is required. It is common practice in a screening experiment to test many new materials, perhaps 20 or 30, at the same time. Since most of these are usually fairly useless, the majority of treatments could perhaps be regarded as providing a large control group; then one could look for individual results that stand out from the rest. Unfortunately, this approach is not always safe, since there is a tendency to test groups of related compounds simultaneously, and from time to time it happens that most compounds in a group are active. For this reason we always use a separate control group that is larger than any of the individual test groups, but still a rather small proportion of the total number of animals in the experiment.

An interesting and important question is whether we can determine the rules of procedure, that is, sample sizes and criteria for acceptance, in such a way as to minimize the financial cost of the whole operation. This problem has been considered by O. L. Davies* of Imperial Chemical Industries, Ltd., London, England, and by J. T. Litchfield and Charles W. Dunnett of Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y. Assumptions must be made about both the relative cost of different parts of the operation and the distribution of effectiveness of the incoming materials. For instance, Davies considers the situation in which a high proportion of materials have zero effect and the minority have a certain positive effect, assumed to be known and constant. For the purpose of selecting a given proportion of materials, perhaps 1 in 100, having a minimal mean cost per effective material selected, Davies shows that a 2-stage procedure, that is, one in which a material may be submitted to a second test if necessary, is more efficient than a single-stage procedure, but less efficient than a fully sequential plan in which the number of stages is unlimited. He suggests that a 3-stage procedure would be almost as adequate as the sequential plan.

Because the assumptions made here are extreme, one may be unwilling to rely too heavily on them. An alternative and simpler approach is to postulate some specific degree of discrimination between materials of various qualities that we should like to achieve. That is, we could specify the probabilities of acceptance for various degrees of activity, which is called the "operating characteristic," and then produce as economical a scheme as possible with these properties. It may be that another operating characteristic would be better, but we could tell this only if we knew all our costs and the nature of the incoming materials; nevertheless, such a plan would achieve the requirements we have set with reasonable efficiency. The most economical scheme in this case will depend on the unknown distribution of activity. We have constructed a 3-stage scheme in which materials may be rejected at any stage, but are accepted only after stage 3. TABLE 1 and FIGURE 1 give details of this scheme, which is indicated as scheme A, and also of a similar but less severe scheme, B. Scheme B requires far more retesting than does scheme A.

We have recently computed characteristic operating curves for 2-stage sequential systems for possible use for labile materials. A 2-stage system involving a first-stage rejection level of 0.45 and a second-stage rejection level of 0.20 yields a curve close to curve A in the 3-stage scheme. This 2-stage scheme gives a flatter curve for low T/C values, that is, it should reject more "good" materials than the 3-stage system for the same acceptance levels of

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* O. L. Davies, "The Design of Screening Tests in the Pharmaceutical Industry," a paper read at the 1957 meeting of the Biometric Society, International Statistical Institute, Stockholm, Sweden.

TABLE 1

RULES OF PROCEDURE FOR TWO 3-STAGE SCHEMES FOR AN ANIMAL SCREEN OF ANTICANCER AGENTS AGAINST SOLID TUMORS*

	Scheme A	Scheme B
After stage 1, reject if T/C > Otherwise, continue to stage 2.	0.54	0.60
After stage 2, reject if $(T/C)_1 \times (T/C)_2 >$ Otherwise, continue to stage 3.	0.20	0.30
After stage 3, reject if $(T/C)_1 \times (T/C)_2 \times (T/C)_3 >$ Otherwise, accept.	0.08	0.15

* Scheme A is used at CCNSC; scheme B is a similar but less stringent plan. At each stage the test material is compared with a control, and T/C is the ratio of mean tumor weights in the test and control groups.

false "positives." This loss of efficiency is of the sort that Davies discussed in considering 2-stage and 3-stage sequential systems.

Since most materials will be relatively inactive, the extra testing contributed by the second and third stages will not amount to a high proportion of the total, and the scheme should be fairly near the optimum for this operating characteristic. Fortunately, it also gives us the largest amount of information about the materials in which we are most interested, that is, the most active ones.

Human Screening

Preclinical and human pharmacology will eliminate many of the materials passed by the animal screens, and those remaining may be considered for possible clinical trial. An authoritative evaluation of a therapeutic substance

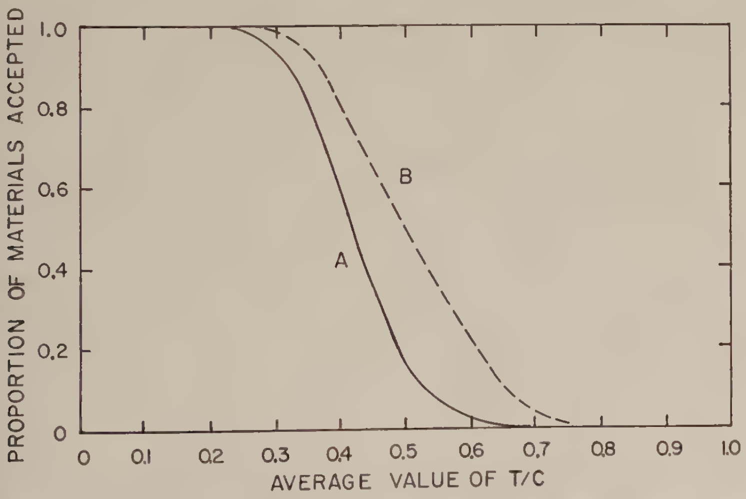

FIGURE 1. Three-stage scheme, A, contrasted with a less severe 3-stage scheme, B. T/C is the ratio of mean tumor weights in the test and control groups.

requires a controlled clinical trial, but there may be more materials available than there are facilities for testing them fully, in which case further screening would be needed. It may, indeed, be good strategy to reduce the severity of the animal screen to such a point that further screening on humans is needed.

Some of the cooperative trials now in progress on cancer chemotherapy are effective screening procedures since, with as few as perhaps 20 patients in each of the test and control groups, one can hardly do more than detect exceptionally good or exceptionally poor materials. The design of clinical screening procedures requires more consideration than it has attracted thus far. Consider two possible designs: one with an equal number of patients in the test and control groups and the other with no controls. Again, the optimal strategy depends on unknown factors. If there are appreciable and unpredictable variations in response due to changes in the severity of the disease of the patients or in the standards of assessment, the controlled design will be preferable, for the usual reasons. If conditions are considerably more stable, the uncontrolled method may be preferable. Even in the latter situation some controls may be desirable for the detection of unexpectedly large changes in response.

It will be as useful here as it is in animal screening to test several new materials together against a simultaneous control. C. C. Li has suggested a sequential procedure whereby relatively poor materials are rapidly withdrawn from the study and are replaced by new materials and good materials are retained, thus receiving a fairly full-scale trial. Our colleague R. J. Taylor has suggested a similar scheme in which the relative proportions of patients treated by different materials in successive periods depend on the results obtained to date, poor materials being received by relatively few patients. Probably it could be so arranged that a poor material was rejected on the average after the same number of patients on the two schemes. Li's scheme would achieve this after a shorter period of time, but Taylor's scheme could provide the advantage of comparison with a larger number of simultaneous controls. Again, the choice depends on factors that are partially unknown.

Acknowledgments

We are grateful to Charles W. Dunnett, Eleanor W. Foltz, Abraham Goldin, Gwendolyn L. Hobbs, C. C. Li, John T. Litchfield, Nathan Mantel, and R. J. Taylor for permission to refer to their unpublished work.

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Discussion of the Paper

Frank M. Schabel, Jr. (*Southern Research Institute, Birmingham, Ala.*): Although I am no statistician, as a contemporary biologist I recognize the the large contributions that proper application of modern statistical procedures make to the evaluation of biological data.

In view of the very large numbers of new materials that are presently being screened for carcinostatic activity, statistical evaluation of results aimed at separating the wheat from the chaff is essential, but I believe that the biologists involved in this program would be remiss if they did not attempt to apply critical judgment to the evaluation of some of the materials selected for further testing or discarded as inactive on the basis of established criteria for interest. This statement should not irk the statisticians, because they are fully aware that any selective procedure they devise will yield both false positives and false negatives. In some instances the biologist can, I think, save some potentially very interesting false negatives from being discarded and likewise avoid much nonproductive work on false positives.

Let me give 1 or 2 examples of what I mean. TABLE 1 lists the average tumor weights from 10 identical, randomized groups of mice implanted with Sarcoma 180. If these were 10 test groups and the average tumor weight of controls was approximately the average of the 10 groups, 1046 mg., no possible active materials would be recorded. If, however, the average tumor weight of the controls was the highest in the group, 1608 mg., and the presently used first factor for activity treated/control (T/C = 0.54) was applied, then groups 2 and 3 would be false positives. If, however, any single material inhibited tumor growth by 25 per cent (T/C = 0.75), then the positive-labeled tumor weight changes in TABLE 1 would be considered possibly active (T/C = 0.54), and as many as 9 of the 10 shown could be scheduled for retesting to attempt to confirm these first suggestions of carcinostatic activity.

That this can happen, we know from experience. For example, in one group of 18 experimental beers in which the average tumor size from control mice was 971 mg., 10 showed T/C of 0.21 or less, and 5 of the 10 had a T/C of <0.05. Nine of these 10 were retested, and only one showed a T/C of <0.54.

Similarly, if the control average tumor weight should happen to be low in relation to the true average, then weak actives would become false negatives.

TABLE 1
AVERAGE TUMOR WEIGHT FROM TEN IDENTICAL GROUPS OF SWISS MICE IMPLANTED WITH SARCOMA 180

Group	Average weight change (gm.)	Average tumor weights (mg.)	
		Actual	Minus 25%
1	+0.3	1007	755+
2	−0.8	745	556+
3	−2.5	720	540+
4	−1.9	1162	851+
5	−2.5	1608	1206−
6	−1.6	1126	844+
7	−2.0	1042	780+
8	−1.8	1138	853+
9	−1.7	919	689+
10	−1.5	1002	751+
Average	−1.5	1046 ± 16%	

This is possible whenever a number of test groups show average tumor sizes considerably in excess of the control average. For example, we recently saw a group of 17 experimental antibiotics in which 12 of the 17 showed average tumor sizes in excess of 140 per cent of the control. This group probably can be dismissed, but the ones with T/C of <0.90 may have had T/C of <0.54 under different circumstances. This type of possible experimental anomaly could result in the inadvertent discarding of potentially useful materials.

I believe there is a serious defect in the present evaluation system, particularly with respect to experimental beers. The present system requires T/C first test = 0.54 or less, T/C first test × T/C second test = 0.20 or less, and T/C first test × T/C second test × T/C third test = 0.08 or less, for acceptance as a possible active material for further study. These experimental beers are natural products; the active principle in any one of them may be unstable, and it is certain to be unstable in some. This required activity sequence in the first sample essentially disregards instability. The present evaluation system also unavoidably overlooks those that have borderline activity in the crude beer. TABLE 2 shows some examples of these that we have seen.

TABLE 2

REPRODUCIBLE ACTIVITY IN ANTIBIOTIC BEERS SHOWING BORDERLINE ACTIVITY

Beer	Average tumor weights, treated/control		
	1st sample	2nd sample	Additional samples
A-748	0.60, 0.62	0.40, 0.51	3rd–26th 7 showed T/C <0.54
A-801	0.61, 0.52	0.98	3rd–31st 12 showed T/C <0.54
A-831	0.54, 0.38	0.86	3rd–13th 6 showed T/C <0.54

To a biologist, these refermentable borderline activities in experimental beers are of greater interest than one that shows spectacular activity in a single test, but which can never be repeated. The biometrician cannot hope to salvage this group of borderline actives unless he sets the screen to yield an unworkable number of false positives; neither has the screener a way of selecting them. If this group is to be salvaged, and I believe it should be, if at all possible, we shall have to rely on the supplier who often has recorded activity in other biological systems with these borderline active materials, which sustains his interest in them.

I believe that maximum effectiveness of the primary screen depends on the application of both the statistician's and the biologist's evaluations of new materials, which should augment rather than oppose each other in achieving the avowed purpose of both.

CHARLES W. DUNNETT (*Lederle Laboratories Division, American Cyanamid Company, Pearl River, N. Y.*): My comments on the excellent paper by Armitage and Schneiderman will be brief. The paper is an interesting contribution to the methodology of screening, which has been attracting interest of late among the statisticians, an interest that will certainly increase in the very near future.

It is worth while, I think, to emphasize the role played by the "operating characteristic curve" of the screening procedure. This curve tells us which levels of activity have a high probability of being accepted by the screening procedure and which levels of activity have a high probability of being rejected. In this sense, it measures the actual performance of the procedure. Whatever rules for acceptance and rejection are adopted, the operating characteristic curve pertaining to these rules can be calculated, and we can then ascertain whether the procedure is achieving our purpose. In developing the Cancer Chemotherapy National Service Center's screening procedure, the authors first decided what performance they required of the procedure by specifying the desired form of the operating characteristic curve, and then determined the acceptance and rejection rules that would meet these specifications as economically as possible. Certainly this approach seems to be a very reasonable one.

Some difficulty may be encountered in reaching agreement among a group of investigators as to the desired performance of the procedure. The chemists who develop the compounds may favor a procedure that accepts compounds of fairly low levels of activity on the ground that these may indicate types of structure they should investigate. On the other hand, the biologists may prefer not to waste time retesting such compounds. All would agree, however, that the procedure should provide a reasonably high probability of accepting any known active compound. As a start, the acceptance rules may be chosen with this in mind, and later changed, if it is found that either too many or too few leads are being provided.

IRWIN D. J. BROSS (*Department of Public Health and Preventive Medicine and Sloan-Kettering Division, Cornell Medical College, New York, N. Y.*): Armitage and Schneiderman wisely inserted a quiet qualification from time to time in their far-ranging survey of statistical problems of animal and clinical screening by using such innocuous phrases as "the choice depends on factors that are to some extent unknown." This raises an interesting question concerning the broad general statements and recommendations that are made in the paper. If, in fact, so little is known about the experimental situation in a given screening operation, and if what actually is known is likely to be markedly different from one screen to the next, then how can we make general statistical recommendations?

My own answer would be this: statistical theory can suggest possible procedures. It can also help us to avoid some very inefficient techniques. Even so, it cannot tell us whether a scheme will work in an actual screen. The only way we can discover this is to test the technique empirically, in actual operations.

Consider, for example, the sequential screening procedure A advocated by Armitage and Schneiderman. In FIGURE 1 of their paper the authors present a graph showing the theoretical performance of the method. This performance is spectacular—on paper. It will almost never pass an ineffective compound or even a compound that produces 30 per cent inhibition. On the other hand, it will rarely miss a compound that produces a 70 per cent inhibition.

The mathematics that underlies the performance curve is impeccable, and we are assured that the assumption used in drawing the curve—that the standard deviation of log T/C from one test to another is 0.13—is approximately true. Can the scientist now proceed to use sequential scheme A in his operations with complete confidence that he will have very little chance of either passing a completely ineffective compound or missing an agent that achieves an inhibition of 70 per cent?

I suspect that the screener who proceeds with such an expectation will have a very unpleasant surprise. In other words, if he takes the precaution of making a pilot run with his new procedure, a step I strongly recommend, I think he will find that he fails to detect known effective compounds far more often than the theoretical performance curve would indicate. The procedure may live up to his expectations, but this would surprise one.

The reason for my skepticism becomes clear if one considers what is implied by the innocent assumption that the standard deviation of log T/C from one test to another is 0.13. What does this mean in terms of the experimental situation? First, it implies that the variability on the log scale does not depend on the percentage of inhibition—that it is the same for a highly effective compound as for an ineffective one. However, there is ample evidence from the Cancer Chemotherapy National Service Center (CCNSC) data on this screen, that variability on the log scale may be far greater for effective agents. In one extensive series the standard deviation turned out to be 0.37, or 3 times the assumed value of 0.13.

To see what this means in practice, suppose we have a compound that produces a 70 per cent inhibition, which the screen is supposed to pick up over 95 per cent of the time. For a standard deviation of 0.37, there is about 1 chance in 3 that this compound will be rejected on the very first trial—that it will not even get a second test (see APPENDIX). In other words, whereas in theory the screen would hardly ever miss an agent so potent, in practice the screen may often miss such an agent; moreover, the screener only rarely comes across agents that can produce so marked an inhibition.

I have pointed out one way in which the scheme might fail but, of course, many other troubles may develop in an actual screening operation. Furthermore, the sequential scheme is only one example; the same objections apply to any theoretical performance curve for statistical methods.

My main point is this: It is dangerous to make general recommendations about statistical design or analysis on a theoretical basis, and especially for cancer chemotherapy screens, for each screen has its own special problems. Statistical techniques must be tailored to fit the situation. This means that a careful empirical analysis of the specific experimental situation is necessary—a tedious, time-consuming, and often thankless task. In other words, the statistician cannot simply sit back and transmit his orders from GHQ—he must make excursions to the firing line to discover what is actually happening there. Mathematics alone will not do the job.

Certainly Armitage and Schneiderman are well aware of this point, but some scientists, and even some mathematical statisticians, are badly confused about it. They tend to believe in a performance chart such as that of the authors' FIGURE 1 because it is derived mathematically, and mathematics, they are convinced, is never wrong! The point is that to derive the chart we must start from a mathematical description of the experimental situation; the mathematics may be all right, but the description may be all wrong. The logic may be impeccable, but if you start from a false premise you may well reach a false conclusion. Statistical methodologies, like any other scientific instruments, must pass the acid test of actual experience before they can be used with much confidence.

APPENDIX

Explanation of how sequential plan A could reject a potent compound (70 per cent inhibition or T/C = 0.30) on the very first trial in 1 out of 3 tests:

(1) Examination of results from several screening contractors for a standard compound used at CCNSC gives a pooled (within contractor) standard deviation of 0.37. This compound is highly effective.

(2) For an average value of T/C equal to 0.30, or −0.523 on the log scale, and for a rejection value of T/C equal to 0.54 or −0.268 on the log scale, the difference between the average T/C and the rejection T/C is 0.255, on the log scale. In terms of a standard deviation of 0.37 this difference is about two thirds of a standard deviation, 0.689.

(3) If T/C were normally distributed, then the probability of an observation falling more than 0.689 standard deviations above the mean would be 0.31, or about 1 chance in 3. Actually, the distribution of T/C is likely to be skewed with the long tail on the right and, as a consequence, the normal approximation would, if anything, tend to underestimate the actual chance of rejection.

(4) The 1 chance in 3 refers to rejection on the first sequential trial, but the total chance of rejection would include also the chance of rejection on the second and third runs, and hence would be somewhat larger than one third.

CHARLES M. MOTTLEY (*Chas. Pfizer & Co., Inc., Brooklyn, N. Y.*): The analogy of the prospector searching for gold is appropriate for our discussion. We are all working over the gravel from a number of streams with little assurance that they contain something of value. It would seem prudent not to spend too much time measuring the size of the particles when we are really interested in finding the "color" that may be left in the washing pan. Our problem is one of reducing the amount of sand and gravel in the pan; then, if there appears to be any "color," we must make sure that it is not iron pyrites, fool's gold.

I have taken a somewhat different view from that of Armitage and Schneiderman as to the best strategy for a mass screening program. I believe that the purpose of the program is not, as expressed by them, to separate the available chemotherapeutic candidate substances into a "majority," which is to be rejected, and a "minority," which is to be retained for further investigation; rather, its purpose should be to discover those compounds, if any, that show a significant carcinostatic effect under test conditions, as a preliminary to further trials, keeping the number of false clues to a minimum.

It must be assumed that we do have suitable criteria upon which to base our judgment as to whether a compound has produced a significant carcinostatic effect. If we cannot agree on such indicators as measures of effectiveness, then the application of biometric procedures, however sophisticated, will be of little help in reaching our objective. In other words, we must agree on how to recognize the "color" of gold when we see it. I have assumed that the inhibition of the tumor growth as revealed by the various screens does provide a useful indication of possible carcinostatic activity and, furthermore, that we can recognize the fool's gold of chance variation.

However, I also take the view that, in a program aimed at the actual discovery of potential carcinostatic compounds, no level of biological activity can be established in advance as a basis for discrimination. It is not unreasonable in this case to expect our gold to appear in the form of fine dust, as well as in nuggets of varying size. If one rules out the concept of a predetermined level, such as the somewhat arbitrary 46 per cent inhibition that has been suggested to distinguish the "good," or interesting, from the uninteresting candidates, then screening procedures based on quality-control concepts currently in use may be open to question. In short, I believe that the problem here is not one of estimation based on an arbitrary standard of comparison, but rather one of tests of significance.

We have examined thoroughly the data accumulated on the subject over the past year at the laboratory of the John L. Smith Memorial for Cancer Research, Maywood, N. J. With regard to the HS 1 and the HEp 3 screens and the S 180 screen, we find that there may be significant unpredictable variations in tumor growth: (1) from one experiment to another, (2) in animals from different sources, (3) in the batches of tumor from different donors, and (4) in the experimental errors of a series of experiments.

These findings lead us to question whether the T/C ratio is the best statistic to use for this work. We feel that the situation calls for a more complicated design of the experiments than is required by the specifications, in order to provide adequate control and valid comparisons.

We are using a randomized block design consisting of 8 blocks and as many as 14 treatments, plus 2 controls in each. The appropriate analysis of variance is then employed to derive the evidence. The test of significance is based on what is known as a one-sided t test. Essentially, we are using the null principle and the concept of the self-contained experiment to help the investigator sort his candidate substances into the following 4 classes.

(1) If the evidence clearly indicates no significant activity, that is, a probability greater than 0.10 of chance variation, he would be advised to drop the substance from further consideration.

(2) If the evidence indicates possibly significant activity, that is, a probability between 0.05 and 0.10, he might decide to defer judgment, store the substance, and test it later when there is less urgency.

(3) If the evidence indicates that a significant effect has occurred, that is, a probability between 0.01 and 0.05 of chance variation, he might decide to run another test to obtain confirmatory evidence.

(4) If the evidence reveals highly significant activity, that is, a probability less than 0.01 of a chance occurrence, he would be justified in concluding that a definite reduction in tumor growth had occurred; he might decide, therefore, that the substance should be developed immediately for clinical trials. As a precaution, he could run an additional test, while the preclinical work is getting under way, in order to obtain further assurance that the substance is really effective.

The rules for reaching the action decisions, namely, whether to drop, defer, confirm, or develop the compound, are based on statistics with well-known distributions and tests of significance that are familiar to biometricians. The option to defer further testing in certain cases permits the investigator to adjust his work load to the situation. The decision to confirm by retesting any interesting compounds permits him to eliminate false clues. We believe this system will provide the most economical use of resources.

The only advantage of the T/C ratio in a screening procedure based on quality control concepts is its apparent simplicity. We may be overlooking valuable leads by using a certain procedure simply because it can be easily carried out by laboratory technicians. The randomized block design and its appropriate analysis of variance are more complicated, but they do have the virtue of leading to valid tests of significance. In order to ease the computational burden, we have programmed the IBM 650 to process the data and provide us with all the values required for interpreting the results. The analysis of the data from one experiment requires about 20 minutes to punch the data on cards and set up the instructions for the machine, and less than 5 minutes to run the actual computations. This includes a transformation of the original observations of the tumor weights to logarithms, calculations of the missing values, and the computations required for the analysis of variance and the tests of significance. Preliminary estimates reveal that the computer and an experienced statistical clerk would cost about the same for analyzing the data from a single experiment. However, because of the setup costs for the computer, which are fixed, and the low incremental operating costs, the IBM 650 is more economical whenever more than 1 experiment is processed.

We believe that it is important not to overlook the possibility of discovering fine gold. I have seen our chemists take a compound that, on the initial tests, showed significant activity but would have been rejected by the system proposed, and transform that compound into one that consistently gives more than 70 per cent inhibition. In other words, fine dust can be transformed into bricks of gold. We believe, therefore, that we should not adopt a screening procedure that would reduce our chances of discovering important leads.

Marvin Schneiderman (*Cancer Chemotherapy National Service Center, Public Health Service, Bethesda, Md.*): We have a division of labor for our sequential plan. Armitage is the first stage and Schneiderman the second stage, or "Armitage does the work and Schneiderman fumbles around and tries to answer the questions." To me the comments on our paper are quite interesting and open up certain areas that I think we should consider. First, it is interesting that 3 of the 4 discussants have introduced the problem of variability and have asked what can be done about it. From the point of view of the biologist, Frank Schabel has shown how the control groups varied and that, if you have a high average control group, you are likely to call materials "active," and that, if you have a low control group, you are likely to miss materials. For this reason, we have built into our system an attempt to enlarge the control group to assure ourselves that it firmly anchors our experiments. The control group carries a great deal more weight than any individual test group. Those of you who are operating with the CCNSC system know that when we test 25 to 30 compounds with 6 animals for each, our control group carries about 30 animals. In this way we have a hedge against the erratic control. We may not always succeed in this. I frequently suspect that we do not, but our system is an optimal attempt. In constructing these systems, the variability of response in the same material tested at another time is considered, with the result that such variables are constantly taken into account in constructing our operating characteristic curves.

The comments by Bross touch a delicate point. I think he is right, but I hate to admit it. One statistician's admission that another statistician is right is against the union rules. For materials roughly down in the range of a T/C of 0.2, we should expect a "miss" on the order of 1 per cent of the time or less. I find that we are apparently missing more than 1 per cent. There are probably variabilities in the system that we have not yet been able to isolate and put on paper. I think the true operating characteristic curve may be flatter than our mathematics has predicted. If it is, we shall have to do something about it.

This, of course, relates to Dunnett's question: "Where are we to put this system?" If we find that our curve is really flatter than the mathematics predicts, this will affect what our system produces. If our curve is flatter, then we can have both too many false positives and too many false negatives.

Mottley's comments are very interesting and they will cause us some work. Our big computer is now operating, and we may program some of our problems for it.

With respect to the "yardstick," it must be pointed out that there is a philosophic disagreement. I think there *is* a yardstick. I think that these screening systems are, within certain limits, sufficiently stable to make it possible for our procedures to be based on experience. If we are able to assume that Sarcoma 180 behaves in a given way most of the time, and if we are willing to take the risk that it may not always behave this way, we shall obtain far more information per trial than from one self-contained experiment. This, of course, is the essence of industrial quality control.

This yardstick is really not as firm as the yardstick at the Bureau of Standards in Washington, D. C., and, without our knowing it, somebody comes in now and then and stretches or shortens it. We must look at it again, to see whether it is the same length as it was when we began to use it. This is in part what Armitage meant by stating that the choice of a system should be under continuous review. The stable or unstable behavior of the system is involved in the final choice. That is our problem with the yardstick, and I must say that we have not checked it as often as we should have.

AN EXPERIMENTAL DESIGN IN COMBINATION CHEMOTHERAPY

By Nathan Mantel
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Public Health Service, Bethesda, Md.

In recent years there has been a parallelism between the statistical procedures developed by G. E. P. Box[1] in the field of response-surface exploration and the laboratory procedures employed by A. Goldin *et al.*[2] and others[3, 4] in the treatment of experimental leukemia with drug combinations.

The emphasis in the Box procedures is on methods for ascertaining the optimal set of working conditions for an industrial or chemical process and measuring the degree of superiority existing at those conditions. In practice, Goldin's procedures are designed to ascertain the optimal drug combination for some particular schedule of therapy and then to measure the corresponding degree of superiority. The objective of the latter experimental procedures, however, is to obtain data that will cast light on the advisability of employing drug combinations rather than single drugs. The results obtained must then be interpreted in terms of their biochemical and clinical implications. It is not anticipated that a particular combination optimal in treating experimental mouse leukemia would also be optimal clinically. It is somewhat more reasonable to anticipate that, if therapeutic advantages can be gained experimentally by employing combinations, they may also be gained clinically.

Essentially, Goldin treats the varying levels of a drug in a combination in the same way that Box treats the varying values for an experimental condition. In doing so, Goldin departs from his field's tradition of attaching intrinsic merit to the material drug values employed. This disregard of material drug requirements is illustrated in FIGURE 1.[5]

FIGURE 1 demonstrates the existence of an optimal level of a single drug for increasing the survival time of leukemic mice. At lower levels of dosage, mice die more quickly because of insufficient inhibition of tumor growth, whereas at higher levels they succumb to drug toxicity. The efficacy of a drug is measured by the maximum survival time it yields, the observed maximum in a sufficiently tight series of dose levels being taken as a near approximation of the drug's capacity on the schedule employed. This concept was used[6] to compare the antileukemic efficacies of several folic acid congeners without taking into account the comparisons of the levels of treatment required.

Although survival time is used as an end point by Goldin and in the experiments described below, it should be recognized that the end point of interest should change reasonably in a developing program. In principle, the end point of interest should be some integrated value of all the benefits, as well as harmful effects and costs of treatment. The problem of evaluating all these factors can be avoided, however, by noting some dominating effect on which interest can be concentrated. Survival time may be the end point of interest today but, with improving therapy, interest may shift progressively to the percentage of cures, then to the time required to achieve cures, and later to other factors.

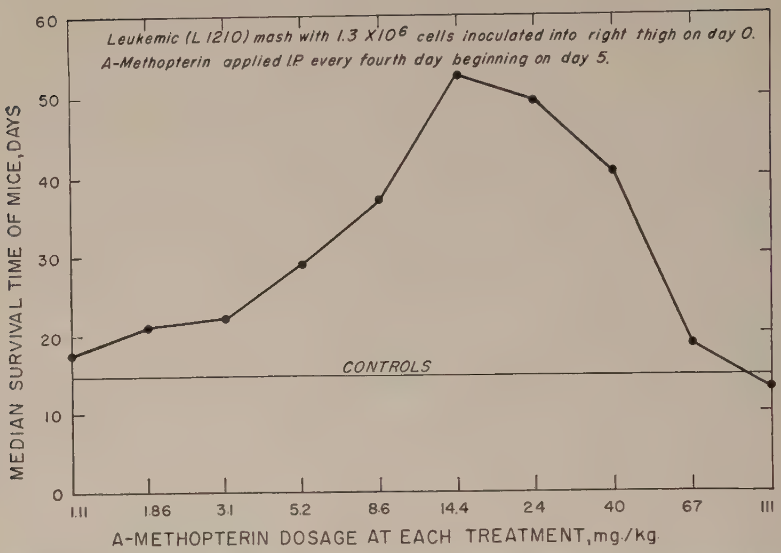

FIGURE 1. Demonstration of the existence of an optimal dose in the treatment of experimental mouse leukemia. At doses below the optimal, mice die sooner because of less effective treatment, while at doses above the optimal they succumb to drug toxicity. Data from Goldin *et al.*, used by permission.[5]

It may be noted that to include the cost of a drug employed in treatment in the full evaluation of that drug would ordinarily lead to the determination of an optimal drug level, even in the absence of drug toxicity. This would occur at the point at which additional drug cost would outweigh any additional benefits it provides.

One could suppose that just as there is some optimal treatment level for a single drug, there is also an optimal treatment level in combination. The problem of testing drug combinations lies, then, in ascertaining and accounting for the existence of such optimal combinations. It is always possible, of course, that the optimal combination will require the use of only one of the drugs employed. In this case, in the sense employed by J. M. Venditti *et al.*[3], it might be said that "therapeutic synergism" does not exist.

FIGURE 2 shows the design of an experiment intended to investigate the efficacy of a combination of 6-mercaptopurine and azaserine (6-MP-AZS) in the treatment of experimental mouse leukemia.* The figure shows 33 experimental groups in addition to untreated controls. The 33 groups were allocated to treatment with each drug alone and to treatment with combinations involving 5 different ratios between the dosage levels of the 2 drugs. A series of geometrically spaced dose intervals was employed for each drug separately and for each of the ratios used.

* This experiment and those illustrated in FIGURES 3, 4, and 5 were performed at the Southern Research Institute, Birmingham, Ala., under contract with the Cancer Chemotherapy National Service Center, Bethesda, Md. The experimental plans were drawn up by F. M. Schabel and H. E. Skipper of Southern Research Institute, together with M. A. Schneiderman and N. Mantel of the Cancer Chemotherapy National Service Center.

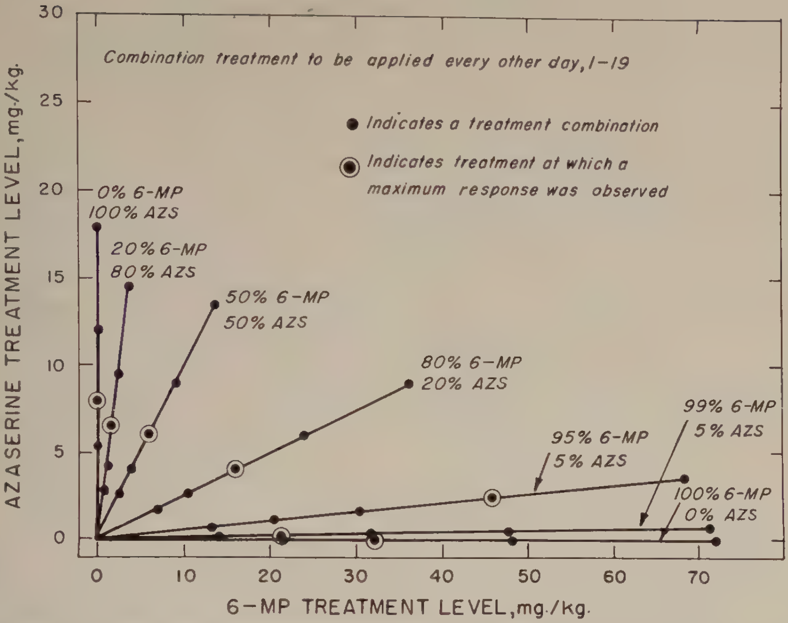

FIGURE 2. Design of an experiment for testing the efficacy of a combination of 6-MP and azaserine (AZS) in the treatment of experimental mouse leukemia. The 33 experimental groups are divided among 5 series employing the agents in various proportions, and 2 series employing each drug separately.

Any analytic procedure appropriate for assaying a single drug is also appropriate for drug combinations in some specified ratio. When, as in the present instance, the analytic procedure consists simply of looking for a maximum, this is perhaps not too important. The Goldin procedure of ascertaining the optimum level and effect for a drug was applied here for each of the relative proportions tested. The encircled treatment groups in FIGURE 2 represent those at which a maximum effect was observed. Within experimental error these tend to define a ridge, the nature of which is discussed below. This approach is distinct from the one commonly employed in investigating drug combinations, namely, that of determining which drug combinations give some specified level of response. The drug combination in this case is deemed useful if it achieves the specified response level at reduced treatment levels.

It may be noted that Goldin *et al.*[2] used essentially the same design in investigating a combination of 6-MP and A-Methopterin. In their study advantage was taken of a temporal separation between drug deaths and tumor deaths to permit evaluation of treatment in terms of the relationship between drug lethality for the host and average survival time of animals not succumbing to drug toxicity. Synergism in this case would be indicated when a better functional relationship was noted for some ratio of application of the two drugs than existed for either drug alone. In that study the analytic procedures employed did make the use of drug combinations in specified ratios essential, but no such synergism was indicated.

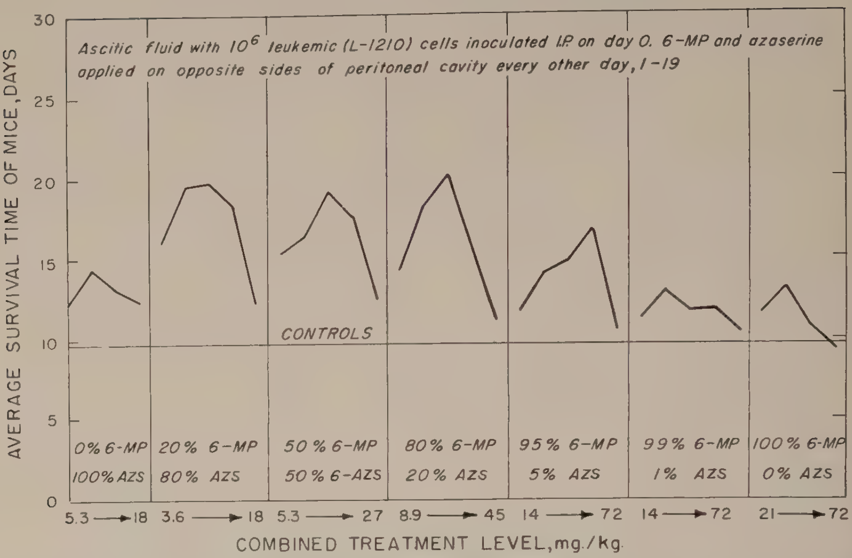

FIGURE 3. Outcome of the experiment design of FIGURE 2. The average survival time for the successively increasing doses in the various experimental series is shown. In each series a maximal average survival time is established.

FIGURE 3 shows the detailed average survival times observed in each of the treatment groups. There is a separate panel for each of the relative proportions used in testing the 6-MP-AZS combination. In each of the panels it can be seen that a maximum average survival time was observed at one of the intermediate levels tested. The average survival time for controls of under 10 days was raised to more than 20 days by one of the treatment combinations. Since neither of the drugs separately achieved extensions of survival time as great as that achieved with a combination of the two, the results obtained may be considered to be a demonstration of therapeutic synergism.

In the left panel of FIGURE 4 the height of the ridge of maximum effect referred to above is plotted. The maximal average survival time obtained with a particular 6-MP-AZS ratio is plotted against the percentage of 6-MP corresponding to that ratio. The therapeutic synergism noted above is evidenced in this plot by the fact that the maximal survival time rises above that obtained with 0 per cent 6-MP and then falls back to the maximal survival time observed employing 100 per cent 6-MP.

The right panel of FIGURE 4 shows the corresponding results obtained in a repetition of this experiment, but with a 5 per cent 6-MP series added and the 99 per cent 6-MP series deleted. The results obtained in this repeat experiment give confirmatory evidence of the synergism already noted. It may be remarked that the dose levels found to be optimal at a particular 6-MP percentage, which are indicated in parentheses in the figure, are substantially confirmed in the 2 experiments.

The nature of the synergism between the drugs employed is unusual in that

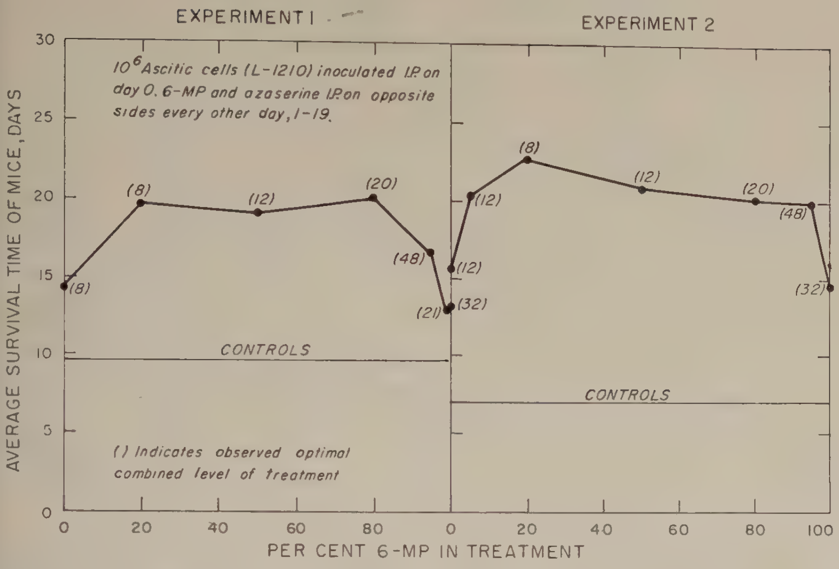

FIGURE 4. Left panel (Experiment 1) shows the profile of the ridge of maximum average survival time corresponding to the data of FIGURE 3. This shows that over a wide range of relative proportions the drugs in combination are superior to the separate drugs. The right panel (Experiment 2) shows the substantially similar results obtained in a replicate experiment.

it is manifest to about the same degree over a wide range of relative proportions, in the present case from 5 to 95 per cent 6-MP. This may have some implications about the cooperative activity of the drugs. If the cooperation involved chemical interaction between the 2 agents, one would expect a somewhat more limited range of proportions over which the combination would be maximally effective. The present therapeutic advantage with the 6-MP-AZS combination appears to arise from their separate therapeutic effects, rather than from any molecular interaction of the agents.

Whether the range of especially effective relative proportions for a combination is wide or narrow, it must generally be true that over some range of relative proportions the efficacy of a drug combination must be intermediate between the efficacies of the separate drugs. The use of a spectrum of relative proportions in an experiment not only provides more information about the activity of the drugs in combination, but also serves to ensure that any greater potential for the combination will be discovered. Employing a single or limited range of relative proportions when testing a drug combination may fail to elicit any existing therapeutic advantage if the proportions tested happened to fall in the intermediate range. This risk was small with the 6-MP-AZS combination, due to the very wide range of proportions over which the synergism is evident.

The demonstration of the superiority of the 6-MP-AZS combination over the separate drugs does not necessarily impute to the combination any especially

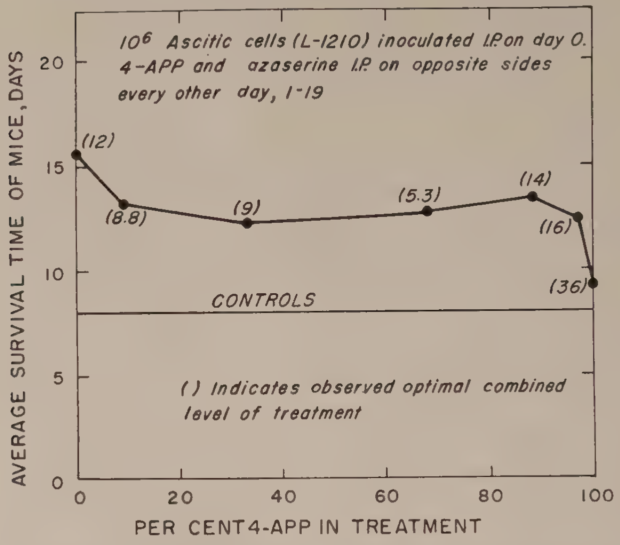

FIGURE 5. Profile of the ridge of maximum average survival time obtained in the testing of an azaserine-4-aminopyrazolo-(3,4-*d*)pyrimidine combination. There is no indication of any therapeutic advantage for the combination over azaserine alone.

important therapeutic effect. Comparable trials with A-Methoperin alone, which were made at the Southern Research Institute, have resulted in somewhat more extensive increases in the survival times of leukemic mice than was provided by any 6-MP-AZS combination.

In contrast to the therapeutic synergism noted with the 6-MP-AZS combination, FIGURE 5 shows the profile of the ridge of maximal survival times obtained with a combination of azaserine and 4-aminopyrazolo-(3,4-*d*)pyrimidine (4-APP) combination. As the figure shows, the maximal average survival time obtained for each of the combinations tested was intermediate between that obtained with the separate drugs. There is no indication of any therapeutic advantage for the combination over azaserine alone.

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Discussion of the Paper

Donald A. Clarke (*Sloan-Kettering Institute for Cancer Research, New York, N. Y.*): The experiment described by Mantel dealing with effects obtained by treating mouse leukemia L 1210 with the combination of azaserine (AZS) and 6-mercaptopurine (6-MP) is reminiscent of contributions to the pharmacological literature by S. Loewe over the past thirty years. Loewe reviewed his work in 1953 on the occasion of a celebration in honor of Otto Loewi's eightieth birthday.[1] He has addressed himself to the challenging problem of how to define graphically the existence of the phenomenon of drug synergism. Incidentally, he has also developed formulas for the demonstration of simple addition of drug effects as well as antagonism. To develop his complicated but graphic isobologram, he records data on a three-dimensional plot. On examining his graphs, one is reminded of the appearance of the sails of a ketch as it tacks into the wind, with data demonstrating synergism producing an "inflated" sail, antagonism a "sagging" sail, and additivity a "tense" sail. The derivations behind these isobolograms are impressive. Similarly, the complex experiment described by Mantel is impressive, as is his graphic plot. However, in consideration of both the work of Loewe and that presented here by Mantel, I am reminded of a wartime slogan: "Is this trip necessary?"

A few years ago my colleagues Frederick S. Philips, C. Chester Stock, and George H. Hitchings and I presented a time-honored pharmacological protocol for examining the effects of drugs used in combination. Rather than using quantal results heretofore employed with this protocol, however, we proposed to employ measurements of growth of the experimental mouse tumor, Sarcoma 180 (S 180), for our quantitative data (table 1).

We proposed to use this protocol in the following manner. As a preliminary experiment, the top third, that is, the X, Y, X + Y, and control groups, would be employed to explore two candidates to learn if any alteration in biological activity might result when the agents were combined; the criteria were either enhanced or suppressed tumor inhibition or increased or decreased toxicity for

Table 1

Protocol to Demonstrate Addition, Potentiation, or Blockade of Biological Activity

Experimental groups (10 mice each)	Treatment and dose*
1	X
2	Y
3	X + Y
4	½X
5	½Y
6	½(X + Y)
7	¼X
8	¼Y
9	¼(X + Y)
10	Controls

* X = optimal or tolerated dose of one agent; Y = a similar dose of a second agent.

the host mice. If either of these parameters was altered, the preliminary experiment was followed by the full protocol.

We believed we could satisfactorily demonstrate drug synergism—or, as we prefer to say, potentiation—if the quarter-dose combination produced results on tumor growth comparable to those achieved by the use of either candidate alone with its full dose. Similarly, we believed we could demonstrate addition if results achieved by the one-half dose combination were comparable to those achieved with either full dose alone. Antagonism, if present, would be evident when the full doses were used in combination.

At the time of our proposal our peers protested that no tumor system could provide quantitative data of the type necessitated by such a protocol. They declared that, at best, our approach was lacking in sophistication. Nevertheless, we did proceed with this approach and, following a pair of observations that a variant of Sarcoma 180 made resistant to 6-MP was sensitive to azaserine and another variant made resistant to AZS was sensitive to 6-MP, we employed this combination in the treatment of normal S 180 early in our efforts.

Results obtained with the preliminary experiment stimulated a prompt follow-up employing the full protocol. Results obtained with the full protocol convinced us that antitumor activity was potentiated, and that, indeed, antitumor activity and host toxicity were separable. When the two agents were employed in the quarter-dose combination, no evidence of host toxicity appeared, but the results on suppression of tumor growth were comparable to results achieved with full doses of either candidate used alone.[2, 3]

In a recent review dealing with "combination" chemotherapy of cancer,[4] Goldin and Mantel considered this observation. They complained that we used these two agents in combination without apparent motivation. In the abstract in which these observations were originally announced,[2] the point made above about the lack of cross resistance of two resistant variants of S 180 was mentioned without further elaboration, for we believed the implications to be self evident. Goldin and Mantel complained further that our results with the combination might have been but fortuitous and, had we done another experiment, the results—especially with respect to host toxicity—might have been different. It is well to emphasize that the experiment has been duplicated many times. The results so obtained have been remarkably uniform. TABLE 2 contains tumor inhibitory data from two different experiments done at different times using our simple protocol and the combination of AZS and 6-MP; the data speak for themselves. Host toxicity also has remained consistently uniform, similar to that reported.[3]

An extension of the original observation was conducted in an effort to learn which agent of the combination was contributing the remarkable property of potentiation. In this experiment, gradient doses of 6-MP and AZS were employed alone to establish base lines. Other animals received the full dose of either inhibitor and gradient doses of the opposite inhibitor. The results of this large experiment are depicted in FIGURE 1 by means of dose-response curves. From these data we were able to adduce little. In the light of the results, however, we were again impressed by the original observations with

TABLE 2

EFFECT OF AZASERINE PLUS 6-MERCAPTOPURINE ON GROWTH OF SARCOMA 180 (DATA NOS. ST-5200 AND ST-5206)*

Therapy†	Per cent inhibition of tumor growth	
	No. 5200	No. 5206
X	42	40
Y	31	46
X + Y	62	63
½X	35	32
½Y	28	28
½(X + Y)	55	61
¼X	25	10
¼Y	23	16
¼(X + Y)	45	41
Controls	0	0

* Treatment of S 180 using the I.P. route; agent(s) given daily × 7 starting 24 hours after implantation.

† X = azaserine, 5 mg./kg.; Y = 6-mercaptopurine, 50 mg./kg.

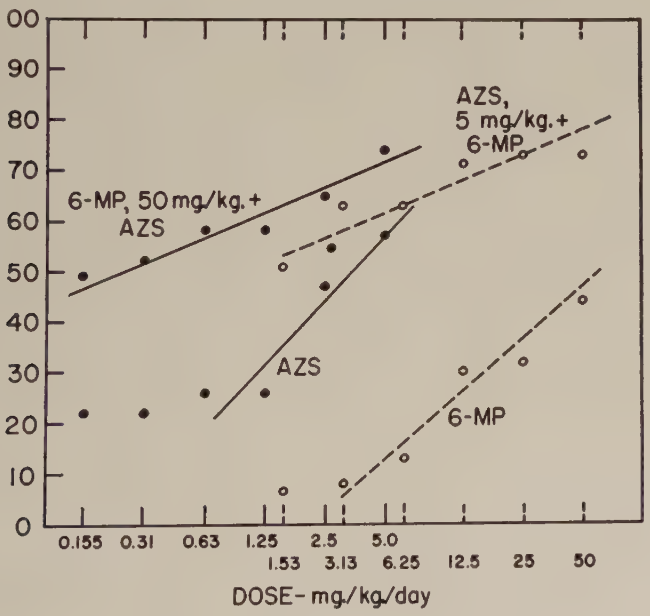

FIGURE 1. Dose response of Sarcoma 180.

respect to quarter doses of the combination. We did observe from the experiment, however, that the azaserine component could be reduced substantially while retaining enhancement of the 6-MP-induced effect of tumor destruction.

In earlier studies we had attempted to treat "established" tumors with this combination of agents and the full-half-quarter dose protocol. We had found host toxicity was so increased as essentially to negate any value from the treatment; the best results, as assessed by recovery from tumor, were no better than could be obtained with 6-MP alone. In our original report on 6-MP as an antitumor agent[5] we pointed out that there appeared to be summation between toxicity to host due to 6-MP therapy and that due to tumor growth. It seemed reasonable to accept the same explanation for failure of the combination in treatment of established tumors. With the information that rather tiny doses of AZS could substantially enhance the tumor-destructive capacity of 6-MP, we selected, for the treatment of established tumors, a 30-mg./kg./day dose of 6-MP which, by itself, would have little or no effect. To this dose of 6-MP we added a 0.6-mg./kg. dose of AZS and initiated therapy in mice implanted with S 180 96 hours earlier. Tables 3 and 4 provide an indication of the course of events at the end of therapy and thereafter. This adjustment of dosage produced results on established tumors superior to any results achieved with 6-MP alone under these conditions. The critical parameter employed in these experiments was resorptive recovery from S 180. Again, we were able to separate antitumor effects and host intoxication, in this instance under more critical circumstances than when therapy was initiated with the combination 24 hours after implantation.

Employing this protocol, we have demonstrated blockade of tumor-inhibitory activity, simple additivity, as well as potentiation of activity such as that seen with the combination of AZS and 6-MP. Also we have had the interesting experience of enhancing inhibitory activity of one agent with a second agent which, by itself, was without activity.

TABLE 3

EFFECT OF AZASERINE PLUS 6-MERCAPTOPURINE ON ESTABLISHED SARCOMA 180. I

Therapy*	96-Hour av. tumor dia.	At end of therapy	
		Av. tumor Dia.	Mortality
	mm.	*mm.*	
6-MP (30 mg./kg./day)	5.4	11.1	0/10
6-MP (30 mg./kg./day) + AZS (0.6 mg./kg./day)	4.9	8.9	0/10
Controls	5.5	12.9	0/10

* Agent(s) injected I.P. daily ×7, starting 96 hours after implantation.

TABLE 4

EFFECT OF AZASERINE PLUS 6-MERCAPTOPURINE ON ESTABLISHED SARCOMA 180. II

Therapy*	7 Days after completion of therapy	
	Av. tumor dia.	Mortality
	mm.	
6-MP (30 mg./kg./day)	9.4	3/10
6-MP (30 mg./kg./day) + AZS (0.6 mg./kg./day)	6.6	0/10
Controls	13.1	2/10

As noted for Part I (TABLE 3).

TABLE 5
AN ALTERNATIVE PROTOCOL

Experimental group	Treatment and dose*
1	X
2	Y
3	X + Y
4	½X + Y
5	X + ½Y
6	¼X + Y
7	X + ¼Y
8	Control

* X = optimal or tolerated dose of one agent; Y = a similar dose of a second agent.

A constructive criticism offered early in our use of the protocol was that it did not take into consideration the possible agent that might have a threshold of effectiveness below which no antitumor activity could be elicited. To prepare for this eventuality we adopted a second protocol (TABLE 5), to be employed should it appear that we were dealing with an agent of which such a threshold effect was characteristic. Using the original protocol, we soon learned that A-Methopterin was one such compound and, in our subsequent studies with this folic acid antagonist, the alternate protocol was used.

In an attitude of biochemical naiveté we entertained one postulate to the effect that, since tumor cells seemed unusually sensitive to folic acid antagonists, perhaps synthesis of this vitamin might be taking place in tumor systems at the cellular level. While entertaining this postulate, the possibility was advanced that sulfonamide therapy (antagonistic to incorporation of the *p*-aminobenzoic acid moiety into the folic acid molecule) given concomitantly with a folic acid antagonist might enhance inhibition of tumor growth ascribable to the latter. From time to time, sulfadiazine, by itself, had produced borderline inhibition of S 180; accordingly, this sulfonamide seemed a likely candidate for exploration of possible enhancement of tumor growth inhibition by the folic acid antagonists. In preliminary experiments sulfadiazine proved to be a potent stimulant of A-Methopterin activity in the therapy of S 180. Of interest was the fact that good inhibition of tumor growth, as well as host intoxication, seemed to be dependent largely on the nutritional state of the host mice. In fact, the threshold effect of A-Methopterin could be demonstrated only when the mice were growing rapidly, hence ingesting more than sustaining amounts of food. TABLES 6 and 7 illustrate enhancement of tumor inhibition and host toxicity by the addition of sulfadiazine to therapy with varying doses of A-Methopterin. In TABLE 7 it may be seen that the mice were actively gaining weight, and therein may be seen gradual failure of inhibition of growth by A-Methopterin as the dose was reduced—even in the presence of sulfadiazine; this is in distinct contrast to the data of TABLE 6, where the tumor sizes are small and quite comparable irrespective of the doses of the two components. A variety of other experiments has led us to the conclusion that this sulfonamide-induced enhancement of A-Methopterin activity may be explained on the basis of suppression of microbial flora of the gastro-

TABLE 6

SULFADIAZINE ENHANCEMENT OF A-METHOPTERIN (A-METHOPTERIN THRESHOLD EFFECT)*

Therapy†	Av. tumor dia.	Av. weight change	Mortality
	mm.	*gm.*	
X	7.7	−1.0	—
Y	6.7	+0.5	—
X + Y	3.6	—	7/10
½X + Y	4.6	+1.0	—
X + ½Y	4.5	+0.5	4/10
¼X + Y	4.6	+1.5	1/10
X + ¼Y	4.2	−1.0	3/10
Controls	10.7	+1.5	—

* Treatment of S 180 using the I.P. route; agent(s) given daily ×7, starting 24 hours after implantation.

† X = sulfadiazine, 500 mg./kg.; Y = A-Methopterin, 1.5 mg./kg. (Experiment No. 1, 2/16/54).

TABLE 7

SULFADIAZINE ENHANCEMENT OF A-METHOPTERIN (A-METHOPTERIN THRESHOLD EFFECT)*

Therapy†	Av. tumor dia.	Av. weight change	Mortality
	mm.	*gm.*	
X	10.9	+2.5	1/10
Y	8.5	+5.0	—
X + Y	6.0	+4.5	2/10
½X + Y	6.0	+5.0	—
X + ½Y	9.3	+3.0	3/10
¼X + Y	7.2	+5.0	—
X + ¼Y	10.3	+4.0	—
Controls	11.6	+4.5	—

* Treatment of S 180 using the I.P. route; agent(s) given daily ×7, starting 24 hours after implantation.

† X = sulfadiazine, 500 mg./kg.; Y = A-Methopterin, 1.5 mg./kg. (Experiment No. 2, 3/23/54).

intestinal tract of mice, flora that presumably contribute folic acid (or a closely related vitamin) to its host. A relative deficiency in the folic acid system thus may be created that becomes manifest when a folic acid antagonist such as A-Methopterin is superimposed.

In their review article, Mantel and Goldin did stress one very important point with which I am in complete agreement. Very frequently in the past, results of combinations of agents in the chemotherapy of experimental rodent tumors have been based on weights of tumors removed from sacrificed host mice. TABLE 8 summarizes results on tumor growth of two antagonists of folic acid metabolism used in combination. If the experiment had been terminated at the end of therapy, potentiation between the two agents would have existed. However, continued observation of the treated mice indicated that delayed toxicity led to excessive deaths, largely negating any value of the combination, at least in this system. It should be emphasized that studies of

TABLE 8

EFFECTS ON SARCOMA 180 AND ITS HOST OF 2,4-DIAMINO-5-(3′,4′-DICHLOROPHENYL)-6-METHYLPYRIMIDINE COMBINED WITH A-METHOPTERIN*

Therapy†	Av. tumor dia.	Mortality	
		End of therapy	7 Days later
	mm.		
X	8.6	—	7/10
Y	8.3	2/10	5/10
X + Y	2.3	9/10	10/10
½X	10.7	—	5/10
½Y	11.8	—	2/10
½(X + Y)	5.8	5/10	9/10
¼X	12.8	—	3/10
¼Y	12.4	—	1/10
¼(X + Y)	8.5	1/10	6/10
Controls	12.2	—	2/10

* Therapy by the I.P. route; agents given daily ×7, starting 24 hours after implantation.

† X = the pyrimidine, 5 mg./kg.; Y = A-Methopterin, 1.5 mg./kg.

this type should be accompanied by observations on the host animals as well as on tumor growth.

In conclusion, may I reiterate that my immediate reaction to the protocol presented here by Mantel is that it is indeed fine to have one's observations confirmed in such a grandiose manner, but I must raise the question as to the necessity of it. However, there is benefit if these very fine results obtained by this complex protocol serve to convince some who doubted our conclusion that potentiation exists between azaserine and 6-mercaptopurine in the treatment of experimental tumors. Should these results possibly serve to improve confidence in our somewhat simpler protocol, I must conclude that the experiment has served a useful purpose.

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G. E. P. BOX (*Princeton University, Princeton, N. J.*): We have had a statistical design presented by Mantel, and another design—or "protocol" as he calls it—presented by Clarke. This question of choice of experimental design raises some interesting questions. One is continually being led back to the fact that the practical excellence of the design is a function of the value of the information with which one starts. In fact, someone once said that you cannot really design an experiment until after it has been performed. When we talk about a "good design" we should ask, "Good for what?" There cannot be such a thing as a good design in any absolute sense; there is only a good design for a given situation. When people argue about what is the best design they are often in fact not arguing so much about the design as about the nature of the situation.

Applying the terminology used in response surface methodology, the problem might be defined as the study of a principal *response* $e = f_1(x_1, x_2)$—for example, the effectiveness of the drug combination against disease—and an auxiliary response $t = f_2(x_1, x_2)$—for example, the toxicity effect on the patient—as functions of the *variables* x_1 and x_2—for example, the dosages of two drugs, A and B. In practice, no mathematical difficulties arise in extending the problem to any number of variables and any number of responses, but it is easiest to illustrate this for just two variables, as shown in FIGURE 1. In addition to the dosages of drugs A and B, other variables not necessarily involving dosages of drugs might be included, and any number of other responses measuring the various effects of the drug combinations could be considered.

The curved lines in the figure are contours of the "effectiveness surface" and the "toxicity surface." It will be seen that these lines define the joint effect of the drugs in all possible combinations, and that the various measures of synergism are simply measures of particular properties of these surfaces. In general, rather than discuss somewhat arbitrary measures of synergism, it seems preferable to consider the response surfaces themselves.

With this representation, the experimental design will be represented by a series of experimental points in the space of the variables as shown in the figure. For a figure like this, Mantel would have one arrangement of points and Clarke would have another. What we are really trying to do is to place the points

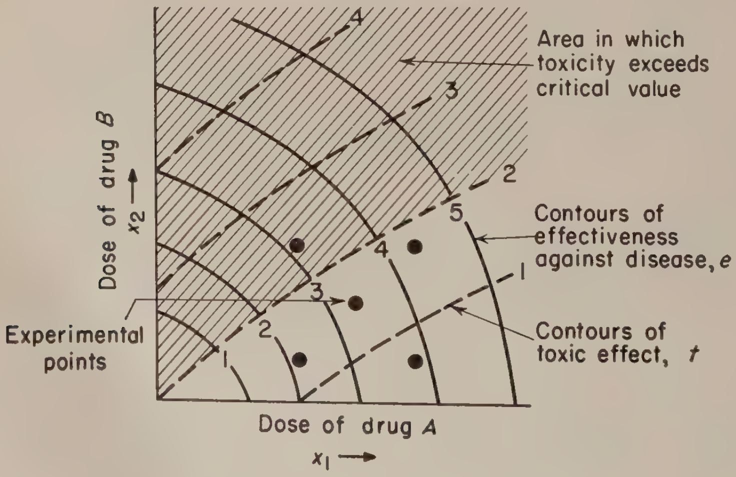

FIGURE 1

so that the surfaces represented by these contours are being explored most effectively.

It will be noticed that, so far as the coordinates of the system are concerned, there is a certain amount of arbitrariness about the diagram. It might be that another experimenter would tend to think more naturally, not in terms of the simple dosages x_1 and x_2, but in terms of certain functions of x_1 and x_2. For example, he might think in terms of variables z_1 and z_2, where $z_1 = ax_1 + bx_2$ was some "combined" dosage of A and B, and $z_2 = x_2/x_1$ was the ratio of dose of drug B to dose of drug A. Plotted in the coordinates of z_1 and z_2, the space would be transformed, and the contours would look somewhat different, and so would the design. One result of this is that a design such as Mantel's or Clarke's could be represented either by perhaps rather curious arrangements of experimental points in the original coordinates or equivalently by a more familiar arrangement in what might appear to be rather curious coordinates. Both would be representations of the same basic design. I think we can see by transformation what was in mind in Mantel's design. If we thought in terms of the proportion of drug A to drug B and the total dose of the mixture given, then in *this* set of coordinates we should obtain an arrangement that would look very familiar—in fact very nearly a factorial design.

What it really comes down to is this: the experimenter tries unconsciously to transform into variables in terms of which the response surfaces are simply expressed. In whatever coordinates one thinks, it is clear that an effective design can be obtained only to the extent that one can predict the type of response relationship that will occur. When we know the shape of the response surface we are trying to "survey," we know where to put the "surveyor's reference points" in order to explore it. In attempting to decide on the best design, it would seem most useful if people would try to write down what they know by drawing some sort of rough surface and then say, "Well now, if we are going to survey this kind of surface, where should we put the points?" It is as simple as that.

There are considerable advantages in looking at things from the point of view of response surfaces. In chemistry, for example, we often want to produce some material in highest possible yield subject to certain restrictions—for example, that it should have a viscosity between certain limits and that its acidity should not exceed a given level. The problem is to find out what conditions of the variables, such as temperature and pressure, we require to achieve this. In this field I think there is a direct analogy with the problem we discuss here. As is pointed out by other contributors, we must consider more than one measure of effectiveness, such as survival time. We wish to achieve increased survival time without, for example, doing permanent damage to the animal or creating a high ultimate mortality. This is analogous to the chemical problem, where increasing a response such as yield to a very high figure is of no use if some other response such as impurity becomes too high.

To solve problems of this kind it is necessary to determine approximately the response surface, not only for the principal response (for example, survival time), but also for auxiliary responses (for example, toxicity effect). The problem is to find a region in which the auxiliary responses are not too high, and then to find the point within this region at which the principal response is maximized. Some problems of this type are discussed in the references cited below.

A further advantage of working in this way is that it facilitates the solution of scale-up problems. We face these same problems in chemistry. People might say, "What is the use of doing research work in test tubes if eventually you intend to manufacture in a large chemical plant?" In the same way, someone might say, "Why are you trying to cure mice? What we want is to cure men." The reason chemical experiments are done in test tubes is that, although they do not exactly simulate conditions in the large plant, they do provide useful approximations. Done from the response surface point of view, the test tube experiments can do more; they can indicate the general shape of the response surface. Usually, because the same basic system is involved, a similar surface is reproduced on the larger scale, although with some distortion. In chemistry the response surfaces on a small scale and on a large scale are similar in much the same way that a mackerel is similar to a haddock: they are both fish. The shape of one can be obtained by a suitable transformation applied to the shape of the other. In the same way, unless there is some basic difference relevant for the experiment for mice and men (some organ found in mice but not in men, for example) similar things should apply to the scale-up problem with animals. Mouse experiments should be regarded, therefore, not necessarily as providing an exact estimate of the optimal combination for man, but rather as giving a general idea of the response surfaces likely to be encountered. Such approximate knowledge of the response surface plays a most useful role in planning the restricted trials possible with human subjects. Consideration of the most effective strategies to be used in experiments of the type discussed here and of the most informative patterns of experiments are listed in the bibliography.[1-4]

Another related subject that could conceivably be of interest in this field is evolutionary operation,[5] a technique used in the chemical industry as a practical way of scaling up from the laboratory to plant scale. The circumstances under which this method is applied again closely parallel the circumstances existing in trials on human patients where usually it is not possible to make more than small changes in the variables, and where experiments that would be quite reasonable on mice in the laboratory cannot be contemplated. The method might in fact be used to get maximum information from the normal treatment of patients by practicing practitioners. It would be necessary for a central agency to obtain agreement that doctors, in using a particular therapy in normal practice (as contrasted with special research studies), would vary the therapy slightly in accordance with a prescribed plan. With a suitable statistical plan, differences arising from small deliberate changes in the therapy can be detected when the information is collected. In this way a steady evolution in medical practice might be set in motion to augment more specialized research studies.

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Frank M. Schabel, Jr. (*Southern Research Institute, Birmingham, Ala.*): For some time I have been convinced (more so, I suppose, than a number of more skeptical investigators) that certain combinations of drugs have greater therapeutic effectiveness in the treatment of several different transplantable mouse tumors than could be shown with either compound alone. Until quite recently the combination chemotherapy trials my associates and I have carried out have been modeled on the earlier work of Clarke and Stock and have consisted of testing identical fractions of the maximum tolerated dose (MTD) of each drug in a combination from the MTD of each drug down through one fourth to one eighth of the MTD of each drug in combination. Perhaps it was not a very elegant procedure, but it consistently yielded results that we interpreted as indicating increased activity of the combination over that observed with either compound alone at double the dose of either compound used in the combination: for example, one half of the MTD compound A + one half of the MTD compound B gave greater inhibition of tumor growth than the MTD of A alone or the MTD of B alone. Similarly, one fourth the MTD of A plus one fourth the MTD of B gave greater inhibition of tumor growth than one half the MTD of A alone or one half the MTD of B alone.

Application of the more extensive combination chemotherapy test procedure designed by Mantel and Schneiderman, which we have been using recently, has yielded valuable information that probably would never have been obtained with the method used earlier. For example, the most effective doses of azaserine (AZS) plus 6-mercaptopurine (6-MP) for the treatment of leukemia 1210 appear to be with combinations of about one half to one fourth the MTD of AZS plus one fourth to one tenth the MTD of 6-MP, such concentrations being significantly more effective than any dose of either drug alone.

Several explanations have been offered for the observed increased anticancer activity of drugs in combination over either drug alone. One suggests that the two drugs in effective combinations block at different sites in the sequence of essential metabolic events in the cell. With combinations of AZS plus 6-MP such an explanation is logical, since it has been shown in both bacterial and mammalian cells that AZS blocks *de novo* purine synthesis, and 6-MP interferes with the incorporation of performed purines into nucleic acids. Another explanation for increased effectiveness of drugs in combination is that tumor cells under treatment become resistant to initially effective drugs, and the drug-resistant cells may be more sensitive to the second agent in the combination; therefore, drugs in proper combination would be expected to be more effective than either one alone. Certainly, leukemic cells become more resistant to 6-MP under continued exposure to 6-MP, but I do not know of any objective data indicating that 6-MP-resistant leukemic cells are more sensitive to AZS than the 6-MP-sensitive leukemic cells.

In any event, we tested the various combinations of AZS and 6-MP against leukemia 1210: first, by treating with each drug alone on alternate days; second, by treating for the first 5 treatments with AZS alone followed by 5 treatments with 6-MP alone; and third, by treating for the first 5 treatments with 6-MP alone followed by 5 treatments with AZS alone. In none of these cases was the observed antileukemic activity significantly greater than that observed with similar doses of either compound alone, and in no case did it approach the antileukemic activity of the most active combinations of 6-MP and AZS. Thus, it appears that demonstration of maximum effectiveness of 6-MP plus AZS combination chemotherapy of leukemia 1210 requires simultaneous treatment with proper doses of both drugs.

Daniel S. Martin, formerly known as Daniel M. Shapiro (*Department of Surgery, Jackson Memorial Hospital, School of Medicine, University of Miami, Miami, Fla.*): Mantel reports an experimental design that is obviously satisfactory insofar as determining the possible benefits of using two drugs in combination is concerned. However, the necessity of assaying such a wide spectrum of doses in such a detailed manner for this purpose is extremely doubtful. A review of combination experiments conducted in our laboratory over the past nine years suggested that the simpler method of combining several regular decrements of the maximum tolerated dose of each drug has been satisfactory for determining effective drug combinations. Although this simpler type of evaluation does not permit the plotting of all the possible "maximal effect ridges," our data indicated that further accumulation of dosage relationships would probably not have produced additional information worthy of the time and effort required.

The above objection to the suggested experimental design is a minor one, and it is subject, perhaps, to debate. However, a major reservation that must be considered in regard to this method is its limitation to evaluating only a two-compound combination. This consideration is of prime importance, since there is clear evidence indicating that the successful chemotherapy of cancer will require combinations in excess of two drugs.

The literature in the field of tissue genetics of neoplastic cell populations reveals that a tumor is composed of a diversity of malignant cell types, each presumably differing in biochemical make-up. It is possible, therefore, that each may require a different chemotherapy. Much of the available literature on this subject has been summarized excellently in a recent article by Hauschka (1957). Figure 1, from that paper, is a diagrammatic representation of some important biotypes in cancer. It seems evident that the wide range of "mutant" cell types present in one individual tumor may demand a correspondingly wide range of therapy.

This multitherapy concept receives additional support from well-documented evidence suggesting that it may be necessary to destroy almost every single malignant cell if recurrence is to be prevented (table 1). Apparently, chemotherapy that leaves residual foci of only a small number of cells will fail to effect a cure.

Current clinical data also argue for combination chemotherapy, particularly multicombination, as the approach to the control of malignant disease. For example, one patient, F. W., developed extensive pulmonary metastases 1 year following a radical mastectomy for breast carcinoma. Within 2 months of receiving stilbestrol therapy there was dramatic disappearance of the massive amount of malignant pulmonary infiltration. The apparently completely negative chest X rays at that time would suggest that the disease was "cured." However, recurrence, no longer hormone responsive, became evident in the patient less than one year later. Had it not been necessary to ablate every, or

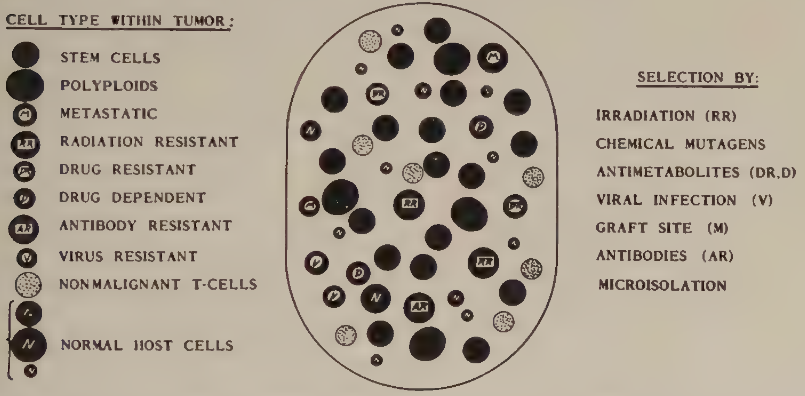

FIGURE 1. Wide range of variant and mutant cell types present within one individua tumor. The cell population is in a more-or-less stable dynamic equilibrium that results from competition between cellular biotypes and selection by environmental or experimental factors. Reproduced by permission of Academic Press, New York, N. Y.

TABLE 1

NUMBER OF CELLS CAPABLE OF TRANSMITTING CANCER

Inoculum (No. of cells)	Injections successful/total		Host	Neoplasm	Source
		per cent			
1	5/97	5	Mouse	Leukemia	Furth & Kahn (1937)
1–48	9/22	41	Rat	Yoshida sarcoma	Ishibashi (1950)
1	46/100	46	Rat	Yoshida sarcoma	Hosokawa (1950)
(cc. of homogeneous suspension)					
0.1	27/28	96	Rat	Walker tumor	Schrek (1935)
0.01	21/39	54			
0.001	12/35	34			
0.0003	2/5	40			
0.0001	0/4	0			

nearly every, cancer cell, this patient should have been cured by the single agent (stilbestrol). That she was not cured undoubtedly indicates the presence of at least some stilbestrol-resistant cell types.

A more provocative case history suggestive of variation in susceptibility to chemotherapeutic agents by malignant cells from the same tumor has been called to my attention by George E. Moore of the Roswell Park Memorial Institute, Buffalo, N. Y. A woman was admitted in March 1957 to the Institute suffering from skull, pulmonary, and pelvic metastases due to breast cancer. The two skull lesions consisted of a parietal and a frontal metastasis. A course of actinomycin D resulted in a gradual disappearance of the parietal metastasis, but a continuing enlargement of the frontal metastasis. By August 1957 the soft tissue extension of the parietal metastasis had completely disappeared, and biopsy revealed no tumor cells. The frontal metastasis had continued to grow rapidly. The patient then received a course of triethylenethiophosphoramide (TSPA), which resulted in dramatic regression of the frontal lesion. The pulmonary and pelvic metastases, however, remained uncontrolled, and the patient expired. Moore has written to me, "In my opinion this illustrates a metastasis sensitive to actinomycin D, a metastasis insensitive to actinomycin D, a metastasis sensitive to TSPA, and metastases insensitive to TSPA." Although it must be admitted that unequivocal proof is lacking, the explanation seems probable.

These two clinical examples, as well as other similar case histories, together with the volume of experimental literature demonstrating the heterogeneity of a neoplastic cell population and the small number of cells necessary to transmit cancer, constitute a formidable array of evidence for the need for multicombination chemotherapy.

It is recognized that the problem of combining drugs is a more complex one than searching for a single agent, particularly if more than two drugs are to be combined. However, this complexity seems an inadequate excuse for ignoring positive evidence suggesting that this may be the necessary chemotherapeutic approach. Complexity should not be the stimulus for reliance upon negative argument and hope that a single agent will be capable of eradicating any type of neoplastic disease.

It is true that the presently available body of facts cannot definitively settle the issue. However, present screening procedures are designed solely to pick up single agents of uncertain "activity," and it is not reasonable to contemplate a successful combination approach based upon combining single agents of unknown mechanism of action and/or structure. The permutations and combinations are indeed troublesome to consider, even if only several dozen such active compounds are reported per year. The multicompound approach cannot be suitably tackled unless screening programs are designed, at least in part, to search deliberately for compounds that lend themselves to this specific approach. At present, provisions for the possible necessity for the combination chemotherapy of cancer have not been included in the national screening program. The facts do not warrant almost complete exclusion of the multicompound approach at the screening level. Reappraisal of our present screening techniques should be undertaken to establish methodology in which both the single and multicompound approaches can be pursued.

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GEORGE H. HITCHINGS (*Wellcome Research Laboratories, Tuckahoe, N. Y.*): Schabel has stated that, by means of the contour map dealing with combinations of 6-mercaptopurine and azaserine, the best combination appeared to be high doses of azaserine with relatively low doses of 6-mercaptopurine. FIGURE 1 (from Clarke's data) shows that, due to the displacement from the right-angle triangle that would be formed by combinations of additive drugs, potentiation does occur. It also suggests strongly that the ideal compound combination consists of a relatively high dose, namely, about four tenths of the minimum effective dose of azaserine, with a relatively low dose of perhaps 0.05 of the minimum effective dose of 6-mercaptopurine.

N. MANTEL: Clarke has referred to the work of Loewe in the field of synergism. Some time ago I had the pleasure of participating with Loewe in a symposium on that subject. As I recall, he defined synergism as any kind of result obtained with a combination of agents. He concerned himself with descriptions of the various patterns and contours of response, such as sails and mountains, which might arise with a drug combination, but in none of this was any specific definition of synergism implied. In my presentation I have also been concerned with the shape of the response surface, but from a rather specific point of view and with a specific definition of synergism in mind. If the combination provides a better therapy than either drug alone, from my point of view this is therapeutic synergism.

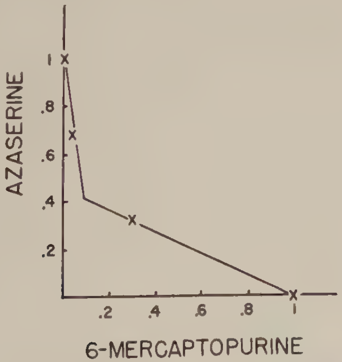

FIGURE 1. Sarcoma 180. Unity on each axis is the amount of the compound that would produce 50 per cent inhibition by weight. Reproduced by permission from *The American Journal of Clinical Nutrition.*

In the results he presented, Clarke employed tumor size as his end point. His data clearly indicated some kind of synergism for the agents he employed with respect to their ability to reduce tumor size. In fact, it was results from experiments of the kind Clarke conducted that led Skipper and Schabel to investigate more thoroughly the azaserine-6-mercaptopurine combination in the experiments reported here.

It should be considered, however, that an antitumor synergism may be neutralized effectively by a synergistic toxicity for the host with no resultant improvement in therapy. Goldin and his associates have reported just this kind of thing in the treatment of mouse leukemia L 1210 with a combination of A-Methopterin and 6-mercaptopurine. There is no essential conflict between myself and Clarke on this subject. It is worth while to find combinations that are especially effective at hitting the tumor. Such combinations may, as in the present instance, provide therapeutic synergism, but not necessarily. The performance of experiments such as those in which Clarke is interested does not eliminate the need for experiments to the point of investigating for therapeutic synergism.

Box, in his discussion, pointed out that the present experiments could be conducted employing a design that could be factorialized through a change of scales. I was aware of this. However, to employ a design that would factorialize exactly would require comprehensive knowledge of the toxic activity of the drugs separately and in each of the relative proportions in which they were employed. Generally, such knowledge may exist only for the separate drugs. In the present instance we hedged against the possibility that the drugs might display a synergic toxic effect for the host by testing at lower levels in combination than when testing the drugs separately.

G. E. P. Box: I think your design is, in fact, almost a factorial design.

N. Mantel: Yes, it is almost, but not quite. Box also pointed out that there may be a host of side effects. The animals may turn blind, or deaf, or display other toxic effects. This I am aware of, as would any biologist working with such a system. What should be analyzed is some measure that fully evaluates the effects of treatment, including such aspects as beneficial effects, lethality, and toxic side effects. This would necessarily be a difficult thing to do. In the present instance I have focused attention on survival time because, as I indicated, I feel it to be the dominating effect. The fact that I select just one effect does not mean, however, that I shall remain blind to other effects that may occur. Nor does it mean that I shall not feel free to shift my attention to some other effects, for example, percentage cures, which may become of dominant interest in subsequent experiments.

In the discussion it was pointed out that the design employed was somewhat complicated. This in itself does not disturb me. For one thing, combinations should not be tested on too routine a basis. We test combinations only rarely, doing so only when there is a firm justification for testing a particular combination. In any such case the testing should be fairly thorough.

If, in some program, combinations are to be tested frequently, then procedures can be devised to reduce the amount of work involved. These procedures may be along the lines Armitage and Schneiderman have mentioned in their contribution to this publication as being employed by the Cancer Chemotherapy National Service Center, Bethesda, Md., in the routine testing of separate agents. The CCNSC procedure evaluates a drug at either some physically maximum dose or nonoverly toxic dose, whichever is lower. This can be adapted to yield a maximum survival time attainable with the drug at levels not exceeding the physically maximum dose while avoiding the necessity for complete drug titration. Testing of a drug combination could be considered equivalent to testing a series of separate drugs, one for each of the relative proportions in which the drugs are employed.

Goldin has pointed out that in his experiments he achieved synergism on some treatment schedules, but not on others. I indicated at the start of my presentation that the design employed had reference to a particular schedule of therapy. Nevertheless, variation in schedule is a complicating factor; clearly, we cannot try all or even an extremely large number of possible schedules.

This complication becomes reconciled when we realize that we are not interested in demonstrating synergism for its own sake. Such demonstration is worthwhile only as it helps point the way to more effective therapeutic procedures. Some variation in schedules may be attempted, with the expectation that useful leads may be obtained. From one point of view therapeutic synergism is only a transient matter, indicating that at some particular moment in time the best therapy known requires employment of the drugs in combination. If some particularly effective way of employing a single drug is subsequently found, the therapeutic synergism may, for the time being, no longer be considered to exist. There is little point in being concerned with so transient a phenomenon as therapeutic synergism. What continues to be of interest is the problem of developing ever-improving therapies.

FACTORS INFLUENCING ANTITUMOR SYNERGISM: RELATION TO SCREENING METHODOLOGY

By Abraham Goldin, Stewart R. Humphreys, John M. Venditti, and Nathan Mantel

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Any account of screening procedures for experimental cancer chemotherapy should include a discussion of screening procedures for detecting the synergistic action of combinations of antitumor agents.

Screening for synergism could be of two general types: synergism pertaining to a single response such as inhibition of tumor growth and synergism pertaining to the interrelationship of two responses such as the host-tumor relationship. The latter encompasses the concept of therapeutic synergism,[1] stressing the importance of the evaluation of treatment with combinations of drugs aimed at improvement of therapy. With reference to the survival time of leukemic mice, for example, therapeutic synergism would exist on treatment with a combination of drugs if treatment with the combination resulted in a more extensive increase in survival time than could be achieved at any dosage with the individual drugs. Both types of screening could be conducted at the biological or biochemical level, either from the point of view of obtaining leads on fundamental mechanism or from the practical point of view of improvement of therapy.

No simple wholly adequate screening procedures for antitumor synergism are at hand. The experimental designs required to establish definitively the existence of antitumor synergism are complex, involving large numbers of experimental animals, trained experimental teams, and extensive physical facilities.

As in the chemotherapeutic screening procedures employing individual compounds, so too in screening with combinations of drugs, compromises must be made with precise methodology in order to shorten procedures sufficiently to make screening practicable. From this point of view, the methodology employed by Stock *et al.*[2] and Skipper *et al.*[3] may at least in some measure provide an appropriate basis for screening combinations of drugs. In this system fractions of tolerated doses of drugs in combination are required to increase the inhibition of tumor growth, to augment the increase in survival time, or to provide more recoveries from tumor than are obtained with maximal tolerated levels of the separate drugs alone. Although this type of methodology may not be wholly precise from a quantitative point of view,[1] it does have the advantage of practicality, since it is simpler in design and employs fewer animals; moreover, the results obtained may be borne out in most cases by more elaborate experiments.

This is well illustrated by results obtained on combination treatment with with 6-mercaptopurine (6-MP) and azaserine (AZS). Several investigators have reported antineoplastic synergism with the combination of 6-MP and

AZS. Skipper[4] reported that in studies employing leukemia L 1210 this combination is synergistic with regard to antileukemic activity in mice. On the other hand, the same combination failed to show any indication of synergism with another leukemia, L 4946, which is relatively resistant to 6-MP. The criterion for synergism was that drugs in combination should be more effective in increasing life span than they are separately at the maximum tolerated dose. Stock *et al.*,[2] employing the fractional-dose requirement for synergism, found that the combination of AZS and 6-MP resulted in synergistic antitumor action. This was evidenced with a combination of each of the drugs at one quarter of a tolerated dose by marked inhibition of growth of Sarcoma 180 and by a 45 per cent recovery from tumor.

In their review paper,[1] Goldin and Mantel confirmed the earlier findings on the increased effectiveness for the combination of 6-MP and AZS against leukemia L 1210; clear extensions in survival time were attained with drug levels that, had they been administered separately, would have been grossly inadequate. Nevertheless, the combination was no more effective in increasing survival time than that obtained with 6-MP alone at sufficiently high doses. However, Goldin and Mantel did not cover a complete range of dosage with the drug combination. They pointed out, moreover, that the proper assay of a combination should include variation in the proportions in which the drugs are employed, as well as variations in levels.

The studies by Schabel and Skipper presented elsewhere in this monograph by Mantel, with early leukemia (L 1210) met the above conditions of experimental design. A series of relative proportions was employed for the 2 drugs, and a series of dose levels of the 2 drugs was employed for each proportion. It was demonstrated that the 6-MP-AZS combination provided therapeutic synergism for leukemia L 1210 over a wide range of proportions of the 2 drugs, that is, the increases in survival time employing the combination of drugs in the varying proportions could not be duplicated by any of a series of dose levels of the 2 drugs employed individually. Thus, the studies presented by Mantel not only corroborated the earlier findings of other investigators, but also extended these findings to include a more detailed description of the relative effectiveness of the action of the drug combination with respect to the host-tumor relationship. It is of interest to note that none of the synergistic combinations was as effective as A-Methopterin alone in increasing the survival time of the leukemic mice.

Studies have been conducted in this laboratory demonstrating not only that the dosage and relative proportions of the drugs employed in combination may influence the demonstration of synergism, but also that the schedule of administration of the drugs may play an important role.

The current studies employing 6-MP and AZS alone and in combination against advanced leukemia L 1210 in mice show: (1) the influence of the schedule of administration of the drugs on the demonstration of therapeutic synergism; and (2) the limitation of host toxicity, as illustrated by treatment of nonleukemic mice. In addition, comparison for relative effectiveness is made with A-Methopterin alone.

Methods

The general methods have been previously described.[5, 6] The two experiments presented were conducted with leukemia L 1210 in 2- to 3-month-old (24 to 30 gm.) hybrid male mice (BALB/c An × DBA/2J)F_1. Stock tumor was carried in the F_1 hybrid male mice for the first experiment and in DBA/2J male mice for the second experiment. In both experiments the tumor inoculum was prepared from spleen mash. Each leukemic mouse was inoculated in the right thigh with approximately 5×10^5 cells contained in 0.1 ml. of a uniform saline suspension. The compound 6-MP* was dissolved in dilute NaOH and adjusted to *p*H 8 to 9 with dilute HCl. Azaserine was dissolved in distilled water. A-Methopterin was dissolved in 2 per cent $NaHCO_3$. The drugs were administered subcutaneously in the axillary region in the constant volume of 0.01 ml./gm./body wt. of mouse. When the drugs were administered concomitantly, they were injected subcutaneously into opposite axillae, the 6-MP always immediately preceding the AZS. Treatment was initiated when the local tumor at the site of leukemic inoculation measured 9 to 12 mm. in diameter, at which time the disease is systemic as well as local.[5, 6] The doses employed and the schedules of treatment for the individual experiments are indicated below.

Results

The two experiments covered in this report are summarized in FIGURES 1 and 2.

The first experiment (FIGURE 1) was concerned with the combined effectiveness of 6-MP and AZS on 3 different schedules of treatment, including treatment every 2 days, daily, and twice daily. Treatment was initiated 8 days following leukemic inoculation, and the median survival time of controls was 11 days.

With 6-MP alone, the maximum median survival time was substantially the same for the 3 schedules tested—16 to 17 days. With AZS alone, the maximum median survival time was 14 days on alternate-day treatment, and rose to 16 days on daily and twice-daily treatment.

The combination of 6-MP and AZS—2 parts 6-MP to 1 part AZS—was markedly synergistic when the drugs were administered every second day, the maximum median survival being increased to 23 days. On a daily schedule the synergism was less extensive, although still evident—maximum median survival time, 19 days—while on the twice-daily regimen synergism was nonexistent—maximum median survival time, 16 days. In the present instance, with treatment initiated only 3 days before the median occurrence of death in untreated controls, the median survival time of 23 days achieved by the combination of drugs on the alternate-day schedule represents a remaining lifetime from the day of initiation of treatment 5 times as great as that occur-

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* For these experiments the 6-MP was provided by George Hitchings of the Wellcome Research Laboratories, Tuckahoe, N. Y.; A-Methopterin was provided by Robert B. Angier of the Research Division, American Cyanamid Co., Pearl River, N. Y.; azaserine (Parke, Davis & Company, Detroit, Mich.) was provided by the Cancer Chemotherapy National Service Center, Bethesda, Md.

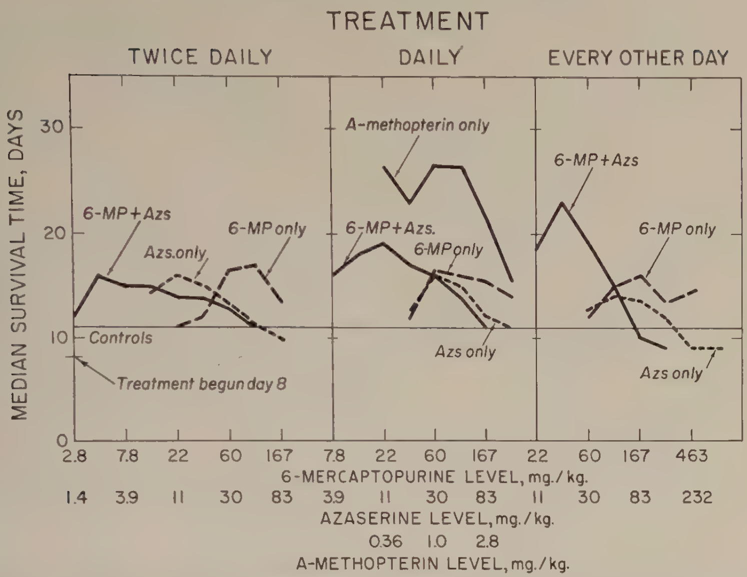

FIGURE 1. Variation in the therapeutic synergism of 6-mercaptopurine (6-MP) and azaserine (AZS) for advanced leukemia (L 1210), with schedule of treatment employed. Daily administration of A-Methopterin alone is still more effective than any of the synergistic combinations. Median survival times shown are based on 8 mice per group, 16 mice for untreated controls.

ring in untreated animals. This synergistic effect, however, was not sufficient to give survival times as great as the median of 26.5 days yielded by A-Methopterin alone on a daily schedule.

With respect to the dose levels at which the maximum survival times are obtained, the following changes were noted. For all groups, as the interval between treatments was increased, the optimal dosage level was increased. However, the optimal average daily dose level increased only for the combination treatment group. It remained somewhat uniform for azaserine alone, and decreased for 6-MP alone.

In the second experiment (FIGURE 2) a comparison was made again of 6-MP and AZS in combination and individually with respect to increasing the survival time of leukemic mice. In addition, groups of nontumor-bearing mice received identical treatments in order to ascertain the toxic lethality of the treatments for normal mice. In combination, the drugs were administered in the same proportion as in the preceding experiment, that is, 2 parts 6-MP to 1 part AZS. The drug A-Methopterin was also titrated for comparison on the same schedule in leukemic and nonleukemic mice. Treatment was begun 8 days after tumor inoculation and was continued every second day through the sixtieth day; only nontumor mice survived to receive treatments beyond the thirty-sixth day. FIGURE 2 shows individual days of death, as well as the median survival times of the treatment groups.

With respect to the question of therapeutic synergism, these results confirm

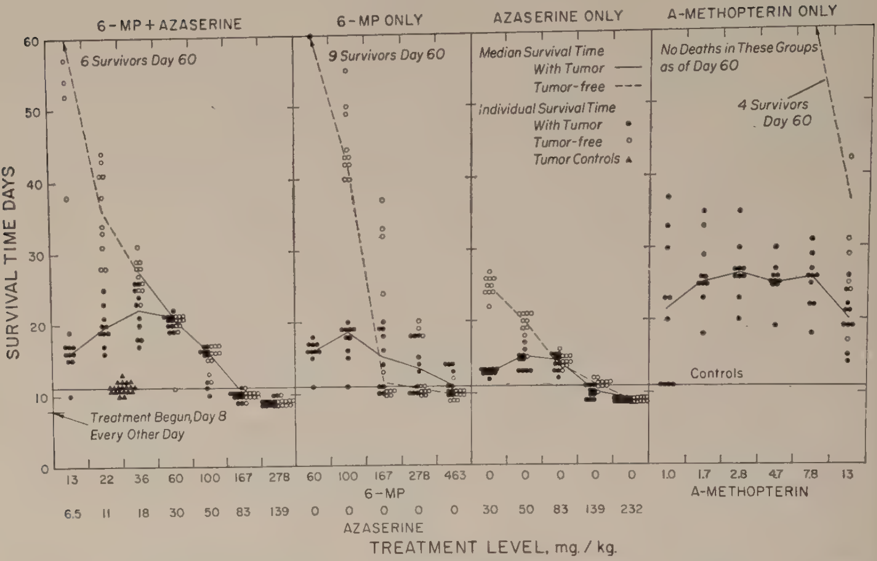

FIGURE 2. Survival of leukemic and nonleukemic mice treated on alternate days with 6-MP and AZS, separately or in combination, or with A-Methopterin alone. Treatments were continued through the sixtieth day, and mice were kept under observation through the eighty-sixth day. No deaths occurred in the A-Methopterin-treated groups subsequent to the sixtieth day. Of the nine 60-day survivors in the 6-MP-treated group (nontumor mice), 6 died subsequently. Of the six 60-day survivors in the combination treatment group (nontumor mice), 2 died subsequently.

those of the preceding experiment in showing the combination to be superior to either drug alone for increasing the survival time of the leukemic mice. However, the synergistic effect was not so extensive as in the preceding experiment. Again, despite the improvement in therapy resulting from synergistic action, the combination treatment was inferior to treatment with A-Methopterin alone.

The data relating to survival time of treated nonleukemic mice are instructive. The optimal therapeutic dose of 6-MP and of AZS separately or in combination was 100 per cent lethal for mice without leukemia. For AZS alone at the optimal dose, nonleukemic mice succumbed relatively early to the drug, all deaths occurring between the fifteenth and the twenty-first days; the next-lower dose level was also 100 per cent lethal, the deaths occurring between the twenty-second and the twenty-seventh days. For 6-MP, in contrast, at the optimal dose the lethal toxicity did not show up until 32 days after the initiation of treatment, all the mice dying between the fortieth and the fifty-fifth days; the next-lower dose level proved 70 per cent lethal, with deaths beginning on the sixtieth day. For the drugs in combination, deaths at the optimal dose were still somewhat early, occurring between the twenty-third and thirty-first days; the next-lower dose level was still 100 per cent lethal, with deaths occurring between the twenty-eighth and forty-fourth days;

while 60 per cent mortality beginning at the thirty-eighth day occurred at the lowest dose level employed.

For all three series the mortality experience at dose levels beyond the optimal was substantially the same for tumor-bearing and tumor-free mice, the median survival times being identical in the case of the combination treatment groups and close together for the AZS and the 6-MP treatment groups.

In contrast to 6-MP and AZS, alone and in combination, A-Methopterin at effective therapeutic doses was relatively nontoxic for nonleukemic mice. Toxic deaths occurred only at the highest dose level employed, and then only to the extent of 60 per cent. Lower doses of the drug showed little evidence of toxicity and no mortality. Four of the 5 nontoxic levels of A-Methopterin showed greater extensions in survival time for leukemic mice than the optimal dose of 6-MP and AZS in combination.

Discussion

In previous studies conducted in this laboratory it was demonstrated that the therapeutic efficacy of a drug is not fixed, but that it may be altered by factors pertaining to the schedule of treatment such as dosage, the interval between treatments, the number of treatments, and the total duration of treatment.[6-9] Other factors, such as the size of the leukemic inoculum, the time during the course of the disease at which treatment is initiated, the administration of a metabolite, and the age and weight of the animal, may also influence the antitumor activity of a drug.[6, 8-13]

By the same token the therapeutic efficacy of a drug combination is not fixed, but may be altered by the manner in which the drugs are employed. The dosages of drugs employed in combination, the relative proportions of the drugs, and the schedule of treatment, all may influence the therapeutic action of a drug combination. Mantel has demonstrated elsewhere in this monograph that 6-MP plus AZS showed antileukemic therapeutic synergism over a wide range of proportions of the drugs. Venditti *et al.*[5] demonstrated that treatment of leukemic mice with a combination of A-Methopterin and 6-MP, when the drugs were employed alone as well as in combination on a daily schedule, failed to result in therapeutic synergism. On the other hand, when the 6-MP was administered daily and the A-Methopterin was administered every 3 days, the combination of drugs elicited more extensive increases in survival time than did the individual drugs. The current experiments showed that 6-MP plus AZS was synergistic for leukemic mice when administered every 2 days or daily, but not synergistic when administered twice daily. As with individual drugs, toxicity for the host may limit the usefulness of drug combinations.

The above discussion suggests that the problems attendant upon screening for antitumor synergism are fundamentally similar to those involved in the screening of individual drugs. If an individual drug shows sufficient activity in a primary screen, secondary screening and detailed investigation may reveal its potentiality. By the same token, if a combination of drugs shows sufficient activity in a preliminary investigation, it, too, may be investigated in detail.

Knowledge of the factors that influence the activity of individual drugs and drug combinations may indeed eventually result in the realization of maximum potentiality for improved therapy. In turn, such knowledge may also lead to improved methods for screening.

Summary

The therapeutic synergism of a 6-MP-AZS combination was demonstrated to be dependent on the schedule of treatment employed. It proved most evident on an alternate-day schedule of therapy, but was still insufficient to make the combination superior to A-Methopterin alone in prolonging the survival time of mice with advanced leukemia (L 1210).

In an experiment exploring the role played by host toxicity in limiting the effectiveness of therapy, it was found that the levels of 6-MP and AZS, either separately or in combination, that were most effective in increasing the survival times of leukemic mice were also invariably lethal for nonleukemic mice. In contrast, the optimal level of A-Methopterin proved to be nonlethal for tumor-free mice.

The results obtained are discussed in relation to the implications for a program for screening drug combinations.

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EXTRAPOLATION TO STUDIES IN MAN

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Some general postulates are in order, lest major goals be neglected through preoccupation with detail. Furthermore, it may be wise to begin with them, since I suspect that there is greater hope for agreement on what the major problems are than there is on the details of their solutions.

Postulate 1. A screen must be judged solely on its pragmatic usefulness for discovering potent compounds. It is one thing to choose a screen because it appears "reasonable" or has theoretical elegance, or to rejoice if by chance some screening technique leads to basic information about a type of tumor; it is quite another matter to equate such a choice with the actual uncovering of useful new agents.

Postulate 2. Potent compounds are those compounds that are potent against human neoplastic disease. The extrapolation to man is not merely the final brick in the screening structure, it is the keystone of the system. A screen that selects hundreds of compounds that are splendid in eradicating animal tumors but useless against human tumors is an unsuccessful screen.

Postulate 3. The screen should be efficient, that is, it should uncover most of the best agents and not suggest as potent many drugs that finally prove useless. In other words, an efficient screen would have a minimum of false negatives and false positives. This raises the delectable possibility of an excess of truly active agents, but an ideal screen would also rank active agents accurately, and would show good predictive ability regarding their relative potency.

Postulate 4. It is impossible to know anything about the correlation between screening data and clinical performance without reliable data on both points. I see no way, for example, to decide about "true" or "false" negatives without testing on man at least some of the compounds that seem worthless for treating animals. One may be unwilling to do this, or it may seem a waste of time, but one should not make judgments about what is or is not being discarded in a screen without at least sampling the discards to see whether the screen's rejections are altogether reliable.

Is such stringent information about negatives always needed? Certainly not. If, for example, our screen reasonably often produces a felicitious number of "true positives," that is, drugs that turn out to be notably active in man, the screen can be pardoned if it is relatively inefficient; and although it may deceive us by calling some good compounds "inactive," or waste our time by calling occasional inert drugs "active," the fact that it does turn up therapeutic bonanzas will probably satisfy us. The question is whether any of our current screens are actually performing in this manner. Moreover, one must keep in mind the record of a screen with regard to "false positives." A screen that is not selecting effective new agents should at least have the decency not to plague us with the necessity of evaluating clinically many compounds useless against human tumors.

A further criterion for an effective screen is the question posed by Gellhorn and Hirschberg,[1] namely, how well would a given screen perform in "discovering" the drugs we already know to be active? Although this is an eminently reasonable criterion, it is subject to certain theoretical and practical reservations. One problem lies in deciding what drugs now available are truly active. This presupposes unequivocal human data of a type somewhat difficult to find in the literature. A second problem is whether a screen that actually can select the drugs we already have is desirable. One could take the position, for example, that the best drugs we have are far from adequate, since they are highly toxic compounds that only occasionally show greater toxicity for tumor tissue than for normal tissue; consequently, a screen oriented around such agents may merely turn up additional compounds of the same unsatisfactory type. The Gellhorn-Hirschberg report[1] can be said to be either encouraging or discouraging, depending on one's philosophic approach, regardless of the performance of a given screen in detecting antitumor activity in "standard" drugs.

From a more constructive vantage point, it seems to me, first, that profitable data may result from a more detailed evaluation of the compounds selected for such evaluation in man. A drug that seems promising by reason of its action in animals deserves a full-scale evaluation in both man and animals. With respect to animals, there should be more data of the kind accumulated by Goldin *et al.*[2]—quantitative measurements of antitumor and toxic effects varying not only the total quantity of the drug and the dosage schedule, but also the age and the type of tumor. No less is needed for evaluating compounds in man.

It seems to me that the optimal schedule for administering a drug to a species is the desirable scheme for ultimate comparison and correlation of data in man and animal; some haphazardly chosen dosage schedule will hardly suffice for a fair trial of the drug. Nor does "pushing a drug to toxicity" mean much if there is considerable discrepancy in toxicity with different dosage schedules. Moreover, in human studies the life span seems less likely to detect oncolytic effect than certain other measurements, such as tumor size. Further, it seems essential, because so many entities are subsumed under the word "cancer," to pit a new agent against a considerable variety of human tumors, and also to study such tumors at various stages of their development.

Could it be that the current predisposition to administer new agents to "last-ditch" patients who have not proved amenable to any other form of therapy is making the detection of drug activity more difficult? A refractory cardiac patient will not respond to such potent agents as digitalis or mercurial diuretics, and a patient almost dead of adrenal apoplexy secondary to meningococcemia is not likely to respond to sulfonamides or penicillin; it is hardly unreasonable to infer that a quasiterminal cancer patient may not respond perceptibly even to a reasonably good antitumor drug. If, for ethical or other reasons, we cannot devise methods for studying oncolytic drugs in patients with early or previously untreated cancer, then perhaps we should attempt correlations of human data only with screening data from animals with advanced neoplasia. Obviously, active drugs are less efficacious in certain tumor systems in animals[2] when the neoplasm is at an advanced stage; we may be asking too much of our screen to test the data collected on early tumors in animals against data collected on advanced tumors in man. Also, I believe that anticancer drugs should be administered to man for a minimal period before being labeled "negative." We need not be surprised if weeks or even months of treatment are necessary before we are able to detect tumor response.

In addition, it seems to me important to pursue certain pharmacological investigations in instances of discrepancies between human and animal data. When a drug seems highly effective against a large variety of animal tumors, but is inert against human neoplasia, we are faced with a troublesome dilemma: Is this discrepancy an indictment of the animal screens, or can it be explained in some other manner? For example, are the results due to unpredicted toxicity in man, which makes it impossible to administer oncolytic doses? Is there any means by which we can avoid or diminish this toxicity in order to permit adequate investigation of antitumor activity? Is the drug metabolized differently in man? Does the human body rapidly metabolize the compound to an inactive form; or, on the contrary, do lower species metabolize the compound to an intermediate compound that is actually the active agent? Such questions, I believe, are at times worth asking.

Finally, we may learn from other fields and other times. An extremely important point is the lack of respect history shows for the sacredness of screening techniques. Not many years ago avian malaria was thought to be an exact correlate of human malaria; since then critical minds and harsh reality have destroyed this tenet. The field of malaria deserves careful scrutiny because it represents a therapeutic area having good quantitative data on both man and animal.[3] Before the development of quantitative techniques in avian malaria, quinacrine was considered by some to be a good antimalarial drug and by others to be inferior to quinine. Quantitative data from the duck and the chick indicated unequivocally that, in suppressant therapy, quinacrine was without doubt superior to quinine, a fact amply substantiated by its behavior in man. There was also fairly good correlation between the performance of a number of other compounds such as sulfadiazine and chloroquine in both man and animal in suppressing malarial parasites. However, even quantitative data in avian malaria were worthless in choosing so-called causal prophylactics against vivax malaria in man, for compounds excellent in chick malaria were inactive in man, and pamaquine, an excellent drug in man, was inert in the chick. In analgesic screening, radiant-heat techniques for producing experimental pain have not been remarkably productive in uncovering new analgesic drugs; but, despite the fact that they failed to detect the only nonaddicting potent analgesic yet discovered, nalorphine, and are unable to demonstrate any analgesic activity in aspirin, these techniques have great popularity in the pharmaceutical industry.

There is a great tendency to carry on scientific love affairs with pet screening techniques, learning with time to disregard blemishes and faults, and developing a paradoxically increasing contentment as vitality and promise wane. My point is that, while we cannot afford the conceit that good cancerocidal drugs will not be found—we must follow Pascal's example in his infinite-gain gamble with regard to the existence of God—there is excellent precedent for the possibility that one or all of our current ways of looking for such drugs may be inadequate.

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Discussion of the Paper

Alfred Gellhorn (*Francis Delafield Hospital, New York, N. Y.*): Lasagna has made some thought-provoking comments about problems in experimental and clinical cancer chemotherapy. Like Lasagna, I have reservations about screening and about clinical trials but, in large part, for different reasons than those he has expressed.

With the expansion of the chemotherapy program there has been a great increase in mechanical screening by techniques outlined by colleagues in biostatistics whose familiarity with the intricacies of transplantable tumors may be as remote as our understanding of mathematical derivations from the Gaussian curve. Results from such large-scale activities may lend themselves readily to IBM* coding and machine analysis, but their real worth in the selection of useful antitumor drugs has yet to be demonstrated.

At the risk of being considered hopelessly antiquated, I submit that the value of a screening program is directly proportional to the thoughtful, critical participation of an experienced tumor biologist whose fingers are frequently soiled by mouse excreta. In such a setup it is more likely that evidence will be more reasonably weighed and that leads pertinent not only to cancer chemotherapy but to pharmacology, physiology, and biochemistry will be recognized and pursued. Exclusive dependence upon a routine screening procedure in which the analysis is largely relegated to a mechanical brain is, in my opinion, unlikely to be more than a wheel-spinning activity.

Lasagna has pointed out the difficulties in translating mouse-tumor studies to man and the frightening prospect that our current screening methods will deliver more of the "limited usefulness" compounds we already possess. This warning may be prophetic, but as yet we have little reason to be pessimistic. When it is recognized that most compounds now used clinically produce only a carcinostatic effect against the experimental tumor, the results in man are perhaps even a little better than in the mouse. The time for real concern will come when a number of compounds that regularly cure mouse tumors are found to have but modest or no effects in human disease. At present there are several tumors in rats that can be cured by alkylating agents. Since we know that this class of compounds, although useful in the clinic, has far-from-dramatic effects, it would seem reasonable to discard the rat tumors for screening purposes.

Lasagna suggests that we are not giving the antitumor drugs a fair chance because we use them only in late stages of the disease; he would have us consider advancing our time of treatment to an earlier point in the natural history of the tumor. This appears reasonable, and yet the difficulty of making critical evaluations of antitumor effect at such early times militates against the plan. I am not convinced that a drug that fails to produce any objective change in the late stage of a tumor is likely to be valuable at an earlier phase of the disease. Treatment of early disease with drugs to improve results is, I believe, wishful thinking of the sort that, if implemented by action, would be hard to test critically because of the chronicity and variability of human neoplastic disease. I hold the same reservations for combination therapy at early stages of neoplastic disease when there is no evidence that such combinations can modify disseminated tumors. Programs of early chemotherapy treatment seem an attractive feature in that they indicate that something new is being done, but the hazard exists that much time will be required to prove them useless.

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* International Business Machines, New York, N. Y.

I should like to mention one last point. Recently in *The New York Times* a news story indicated the large proportion of the total Public Health Service cancer research money being expended on cancer chemotherapy. Of this, the major allocation was made to contract research, chemical synthesis, empirical screening, and clinical testing. This great effort is predicated on the assumption that, if sufficient time, money, man hours, and mice are invested, something immediately applicable to human cancer will emerge. This may or may not prove to be true, but in the allocation of funds it is essential to provide generously for other areas of cancer research that may be less dramatic but may provide more knowledge of value. These include cancer prevention, etiology, and host physiology. Finally, let us reaffirm our conviction in communicating with one another, with Congress, and with private foundations that from basic research on cell biochemistry will come the knowledge for our ever-increasing understanding of normal and uncontrolled growth.

JOSEPH LEITER (*Cancer Chemotherapy National Service Center, Bethesda, Md.*): I am very much gratified that Lasagna presented the "infinite-gain gamble" approach rather than the futility approach. With clinical investigators of such caliber who are intimately associated with the evaluation of these drugs, we must look forward to the better gamble. Borrowing from one of Lasagna's metaphors, I should like also to make a plea that we not have unduly long courtships with our screening methods, that we put them to the test fairly soon, and that we not insist too strongly on immediate parental or familial approval, that is, conformity to accepted screening methods. Sometimes worthwhile marriages occur without parental approval.

FREDERICK S. PHILIPS (*Sloan-Kettering Institute for Cancer Research, New York, N. Y.*): I commend Lasagna for his eloquent, soul-searching appeal that we never become so overly entranced by assays that we refuse to discard them when they have failed. I hope that Lasagna did not start from the premise that either he or anyone else was ever enchanted with mouse tumor assays. Certainly, such enchantment could lead to bitterness when the clinical need is so obviously unmatched by the products of cancer chemotherapeutic screening programs. If Lasagna is bitter—and I am not certain of this—it is an understandable bitterness, for he is responsible for patients whose needs are desperate. He wants therapeutic weapons that are not yet at hand; he deserves to have them. From this point of view he is right in questioning the value of rodent tumor assays. From my contacts with colleagues working in cancer chemotherapy, however, I am convinced that few workers are satisfied with our present assays. Most of us do our best with what is available to us now, and most of us would be delighted to acquire better tools should we become convinced of their greater advantages.

I share Gellhorn's impression that the clinical results obtained with the products of our current assay programs are actually consistent with the laboratory data. I have never been particularly impressed with the effectiveness of most cancer chemotherapeutic agents against mouse and rat tumors. For example, most of the agents are quite ineffective if treatment is delayed by more than a day or two after transplantation. Most of the agents have failed to have demonstrable effects on spontaneous tumors. There is wide variation in the susceptibility of different transplanted tumors, even when optimum conditions are chosen for demonstrating therapeutic effectiveness. Moreover, some of the best demonstrations in experimental tumors, wherein "cures" have been reported, may have been complicated by the involvement of host-immune mechanisms, although we have no reason to suppose at present that immune mechanisms will play any role in the treatment of spontaneous experimental tumors or of clinical cancer.

These are but some of the factors that should be considered in the evaluation of the usefulness of mouse and rat tumors as assays. There is an additional factor that I firmly believe we all tend to overlook in our fervent search for new modes of therapy. In all too many instances we are inclined to ignore the really narrow margins of safety shown by most of the present chemotherapeutic agents, even in experimental tumor systems. With few exceptions, host toxicity is present when agents are given in doses that inhibit tumors. In almost all instances therapeutic indices are negligible. It is certainly not correct to de-emphasize the loss of 1 or 2 gm. in treated mice on the grounds that these values are less than 5 to 10 per cent of initial body weight. If this is the price the host must pay for a significant degree of tumor inhibition, then the cost is too high in human beings. Five to 10 per cent weight loss in patients is obviously intolerable; but what is more serious is that such losses usually are the consequence of grave pathological changes in host tissues. Their presence, in association with disturbing symptomatology, compels the use of doses well below those employed in experimental assays. Even assuming clinical tumors to have susceptibility akin to that of experimental tumors, the demonstration of clinical effectiveness always will be difficult or almost impossible with agents that have negligible chemotherapeutic indices.

I believe it reasonable to assume that our assay systems are capable of detecting therapeutically significant drugs. Until such agents are detected, many disappointments might be avoided if we become more discriminating in what we recommend for clinical trial and if clinicians are more discriminating in their interpretation of laboratory data. I have no doubt that we now have excellent tests for the statistical significance of results obtained in screening programs. However, statistical tests are no substitute for thoughtful judgment about therapeutic significance, which must take account of all the factors just mentioned.

In spite of our different attitudes toward the current status of rodent tumor assays, I do want to endorse Lasagna's emphasis on the importance of learning more about the nature of the specificity of action of chemotherapeutic agents. There are examples of outstandingly good effects achieved by certain compounds in certain experimental tumors. Two that come to mind immediately are the susceptibility of Ca 755 to 6-mercaptopurine, as shown by Skipper's group and by others, and the striking sensitivity of the Ridgway osteogenic sarcoma to the actinomycins, as demonstrated by Sugiura and Stock. These are unquestionably impressive findings. Why are they so limited in scope? Why is the majority of experimental tumors not equally susceptible? Why cannot similar changes be consistently produced with the same agents in at least some solid tumors in patients? The fact that even a few cell types can be destroyed so effectively and selectively indicates the tremendous potential of such agents. Why do we fail to place so little emphasis on the nature of this specificity of action?

Biochemists have learned much about the mechanism of action of cancer chemotherapeutic agents. Indeed, their knowledge of the modes of action of agents such as A-Methopterin, azaserine, 5-fluorouracil, or 6-mercaptopurine is an elegant contribution to both biochemistry and pharmacology. I know of no other families of drugs in which mechanisms are better understood and only a few in which they are understood as well. However, such advances in our knowledge have failed to explain the mechanisms underlying specificity of action of the chemotherapeutic agents; no one can state clearly why it is that Ca 755 is so exquisitely sensitive to 6-mercaptopurine by comparison with other, less susceptible tumors. I maintain, with Lasagna, that we need this kind of understanding, and that it need not be obtained only from studies with nonsusceptible human cancers. Many questions could be answered with transplanted tumors in animals; for example, the mouse implanted simultaneously with a sensitive and a resistant tumor could provide a most instructive setting for many kinds of biochemical studies of specificity of action. I am almost certain that, in time, such studies would contribute to the better use of chemotherapeutic agents in the clinic.

Donald A. Clarke (*Sloan-Kettering Institute for Cancer Research, New York, N. Y.*): I find myself in agreement with Lasagna on some important points. I do believe therapeutic trials of agents found to possess antitumor activity in experimental systems can and should undergo extensive modification. Philips complained recently that, in a way, it was too bad that the nitrogen mustards were the first really effective agents to be found useful in the chemotherapy of cancer. By virtue of the experience gained with them, the principle of "treatment to toxicity" became fairly well established. Accordingly, with most of the agents that have entered therapeutic trial, prolonged administration of nontoxic doses has never been explored. Certainly all of us concerned should ask ourselves with all seriousness why agents that have proved potent inhibitors of tumor growth in experimental animals fail to produce comparable results in human neoplastic disease. Not only should we ask this question, but we should set about finding the answer or answers. It is not improbable, as a matter of fact, that we already possess an experimental system—that of spontaneous mammary tumors in mice—that could shed substantial light on this problem. This is to say nothing of the work that should be done at the human level, to which Lasagna has made some reference.

However, these levels of agreement notwithstanding, I cannot agree with the implication that the present screening methods for anticancer agents have failed in their purpose. These methods have produced results; there has been a flicker of correlation. Man's effort to improve his lot cannot be cast aside simply because man has yet to attain perfection of himself and his environment. True, the concepts about avian malaria proved erroneous, but work with avian malaria produced some worthwhile results. Granted that the application of heat to rats' tails will not tell us that aspirin is a good analgesic, this test nevertheless unearthed some other good analgesics.

What do we do when we set up a screening procedure? As closely as possible at the laboratory level, we try to approximate the human situation we are attempting to alter favorably. We then scrutinize our effort. In the meantime we are gaining experience and learning new things that may prove of direct or indirect value.

Lasagna contradicts himself by noting on one hand that "there is a great tendency to carry on scientific love affairs with pet screening techniques" and, on the other, "an extremely important point is the lack of respect history shows for the sacredness of screening techniques." With the latter statement I can agree; with the former I cannot.

There is nothing sacred about the present techniques used in the search for anticancer agents, the varied nature of the program presented in these pages attests to that. Constant appraisal and criticism are warranted and are being made. All of these approaches suffer from a common lack—the absence of a standard, an agent that will cure human cancer. In the absence of this standard we can do only the best we can. When we possess methodologies that will move us closer to our objective—and we have—it would be ridiculous to abandon them until we have at hand some better plan. In the meantime, we can spend our effort on learning the deficiencies of our current approaches and how to overcome them, how to make our present methods more predictive. We can expend our efforts seeking supplemental methods that will enhance the basic observations made with the present screening procedures and new and better ways to achieve our objective.

FRANK M. SCHABEL (*Southern Research Institute, Birmingham, Ala.*): I wonder if Lasagna has any ideas as to how the testing of drugs that have shown no anticancer activity in experimental animals can be carried out in humans with cancer. I agree it is necessary for proper evaluation of our experimental screening procedures to test in man both actives and inactives from the experimental screen, but the traditional prejudices against experimentally testing inactive materials in man is deep-rooted and hard to circumvent.

Table 1
Chemotherapeutic Indices for Some Known Agents

Agent	S 180	755	L 1210
A-Methopterin	<2	0	4
6-Mercaptopurine	ca. 2	ca. 40	2
N-Methylformamide	ca. 3	<2	<2
8-Azaguanine	0	4	2
Azaserine	ca. 2		2
DON	ca. 2		3
Aminonucleoside	0	2	0
6-Chloropurine	ca. 2	6–8	ca. 2
6-MP riboside		ca. 40	ca. 2
HN2	ca. 2	<2	<2

I propose to discuss briefly Lasagna's constructive point regarding full-scale evaluation in animals and man of agents that appear promising after primary testing in animals. The procedures used by most laboratories screening for new anticancer agents are designed to detect minimal carcinostatic activity against transplantable neoplasms barely established in test animals. Agents picked up in such a sensitive screen are put to test in man at the opposite end of the sensitivity scale, usually in advanced, disseminated, and often terminal cases. Furthermore, we know that agents active against transplantable animal neoplasms often have a low chemotherapeutic index (ratio of maximum nontoxic dose to minimum effective dose) and a narrow spectrum of antitumor activity. Table 1 lists the chemotherapeutic indices of a number of the better known anticancer agents against the transplantable tumors used in the Cancer Chemotherapy National Service Center's primary screen.

If high chemotherapeutic indices in transplantable tumors were of great value in extrapolation of experimental results to probable effectiveness in man, neither A-Methopterin nor 6-mercaptopurine (6-MP) would be of great interest in the treatment of leukemia, yet both have usable temporary effectiveness. The chemotherapeutic index of A-Methopterin against the solid tumors in the screen is low against S 180, and the drug is ineffective against Adenocarcinoma 755. Still, it has been reported to be effective against at least one human neoplasm other than acute leukemia.

We should all be interested in the possible activity against human tumors of any new drug with a high chemotherapeutic index against a tumor used in screening, although to date the chemotherapeutic index has not proved a reliable point of reference for judging probable effectiveness in man.

Often I have heard it said that any drug capable of causing established and advanced animal neoplasms to melt away under its treatment would be in great demand for trial against any and all human tumors. I share this belief, and I further believe that such a drug would have great promise of being effective in the treatment of one or more human tumors. Perhaps we should be seeking, more intensely than we are, a test system for evaluating the effects of new drugs on established animal tumors.

For a number of years Stock, Sugiura, Clarke, and their associates at the Sloan-Kettering Institute for Cancer Research have been evaluating new

TABLE 2

EFFECTS ON MOST SENSITIVE "ESTABLISHED" TUMOR (INHIBITION OF FURTHER GROWTH OR REGRESSION) "CURABILITY"

Treatment	Dosage (mg./kg./day)	Treatment period (days)	Avg. tumor wt. (mg.) and No. of tumors			
			8th day	13th day	19th day	30th day
None	—	—	238 (10/10)	1426 (10/10)	3041 (6/6)	—
6-MP	30	1–12*	—	0 (0/9)	—	0 (0/5)
6-MP riboside	50	1–12*	—	7 (2/10)	—	0 (0/8)
6-MP	30	8–18†	—	—	66 (3/7)	—
6-MP riboside	50	8–18†	—	—	207 (8/10)	—

* Initiation of treatment at 24 hours and continuing through twelfth day will "cure" Adenocarcinoma 755.

† Initiation of treatment at 8 days (tumor weight 238 mg.) will cause some regression (6-MP) and some apparent "cures."

drugs and combinations of drugs not only for carcinostatic activity but also for the ability to cure experimental tumors. I believe they consider these studies valuable in assessing the potential usefulness of new agents.

My associates and I have done some work with established Adenocarcinoma 755 (Ca 755). TABLE 2 lists some results we have obtained with the treatment of established Ca 755 treated with 6-MP and its riboside. Apparently 6-MP and 6-MP riboside can cause some regressions and some cures of this tumor. Perhaps we should try to test new carcinostatic agents picked up in primary screens for their ability to cause regressions and cures of as wide a spectrum as possible of established and advanced animal neoplasms. Based on past experience, the number of new materials showing such activity would be expected to be small, but we might be able to extrapolate activity against established and advanced animal neoplasms to probable effectiveness in man more reliably than has been possible with methods used thus far.

LASAGNA: I agree with Leiter that there should be very short courtships in the screening techniques, and I disagree with Philips. I think it is not a matter of being enchanted or disenchanted with rodent tumors; it is a matter of whether that courtship, for example, may not have gone on for too long. He admits that no drugs work very well in this case. I think that by everyone's agreement no remarkably good compounds have been turned up, and yet the screen continues to be used.

Regarding the statement that no excellent drugs will be missed, I think this may be true. It may, however, be only a pious hope. We have abundant evidence that potent drugs *are* missed in screens. I mentioned two, nalorphine and pamaquin.

Philips says there should be no reason for disillusionment on the part of the investigator. I stress just the opposite point of view. We should be disillusioned, not prematurely, but at a reasonable point, so as to stir people to move in other directions that may be more productive.

With regard to Clarke's statement, I have no argument against any of the screening techniques per se. I cited avian malaria to indicate that it was useful in turning up compounds. It had its limitations, but still was one of those screens that did keep us happy because it turned up chloroquin. With such a performance I think no one cares about false positives or false negatives.

Regarding radiant-heat techniques, I take issue with the statement that they have turned up any interesting new compounds. What they have achieved has been the study of congeners of accidentally discovered compounds, thus turning up others. However, none of these have been significant improvements over the parent compounds.

If Clarke will re-examine my remarks he will find that my "contradictory statements" are not mutually exclusive.

DISCUSSION OF PART VIII

By John T. Litchfield, Jr.

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It is quite clear that a variety of purposes are served by the present rodent screening programs, whether they be those of the Cancer Chemotherapy National Service Center, Bethesda, Md., or those of other independent institutions. It appears that the sequential systems may work very well or very poorly, depending on the behavior of the actual test. It is clear also that one cannot simply calculate how the test should be made and expect it to work. These things must be calculated on the basis of the actual performance the test is giving, and this performance must be re-examined constantly if the yield is expected to be at all close to calculation.

With any of these tests we should be most unlikely to miss a highly active compound. However, great concern has been expressed over the possibility of missing the marginal compounds. Certainly we could design a test that would never miss a marginal compound—with the result that we should then be testing scarcely anything. If we are willing to live with an inefficient test, it is quite possible that, in the end, we shall discover far more worthwhile leads than if we insisted on a highly efficient test.

These screening tests serve a variety of purposes. They tell the chemist whether his compound is active, which is the information he seeks. They reveal new types of biological activity that have gone undiscovered because the compounds have never been tested before. There is no reason to believe that the tests are intended primarily to sort out a large proportion of things of no interest. The concept of no interest is a factor, of course, but the aim of the tests is always to find substances that have sufficient activity to make them worth further investigation.

These tests should not be confused with those used for evaluation. The simple screening tests, particularly the sequential variety, give us nothing more than a very qualitative answer, namely, that the compound tested at some arbitrary dose level is active or not active, interesting or not interesting. We do not learn how active the compound is; the test is not intended to yield that kind of information. Evaluation necessitates a myriad of studies for measuring the activity of a substance already known to be active.

Because a number of substances have been found active, we have become involved in the area of combination therapy. It appears that, in terms of studying combinations to see if they offer what might be called synergism, a number of designs are possible. It appears also that synergism is where you find it; the type of design used may or may not find it. Sometimes it is possible for a very simple design to show a synergistic effect of a combination of two things. On the other hand, a very complex design may disclose a synergistic effect that could easily be missed in a more simple design.

At this stage, when we have so little knowledge about combinations, when we scarcely know how best to use two things at once, we need complex designs, not for discovering synergistic actions, but for revealing the interplay of the many factors involved, such as the toxicity and the side effects. Beyond the fact that synergism has been observed, these complex designs, if properly designed and carried out, can yield a wealth of information.

By developing this broader knowledge of how best to use two things together, we hope to progress to the more complex problem of using three things together. When we face the possibility of using more than three, the problem of handling the complexities will become rather staggering.

With respect to trying to relate to man what we do with animals, clearly much progress has been made. Many programs now in progress will yield a wealth of information in the next few years. At this stage, criticism does not seem worth while. Both in the laboratory and in the clinic we have a very great obligation to acquire better data and learn better what it is we are trying to accomplish and how best to achieve our goal.

Finally, the question has been asked, "Do we know enough about cancer to justify the greatly expanded effort for chemotherapy screening in animals and evaluation in man?" In time we should have the answer to this. At present, most of the discoveries have been made by accident. At least, the great expansion of cancer research in general has created an environment favorable for the happy accident of the discovery of better ways to treat and cure this disease.

STUDIES ON THE USE OF *IN VITRO* PROCEDURES FOR THE SCREENING OF POTENTIAL ANTITUMOR AGENTS: COMPARISON OF ACTIVITY IN MAMMALIAN CELL CULTURES AND MICROBIOLOGICAL ASSAYS ALONE AND IN COMBINATION WITH EXPERIMENTAL ANTITUMOR ACTIVITY*

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Introduction

Previous reports have shown that the majority of agents known to exhibit antitumor effects in animals or man are cytotoxic for human and animal cells in mammalian cell culture[1-3] and are inhibitory in one or more of several microbiological assay systems.[4] The purpose of the present communication is to compare the results obtained with a series of reference compounds in mammalian cell cultures and microbiological assay systems with those obtained with experimental tumors *in vitro*.

Materials and Methods

The agents considered in this and the previous studies[2-4] were a series of 200 compounds from the files of the Cancer Chemotherapy National Service Center (CCNSC). There were 180 compounds in the series which, at the time of analysis, were considered to have been adequately studied *in vivo*, that is, in at least three experimental tumor systems. Data as to the antitumor activity of these compounds were obtained from: (1) published data on activity in experimental tumor systems, summarized in a report from the CCNSC,[5] including, in part, unpublished studies carried out at The Children's Cancer Research Foundation under the auspices of Sidney Farber;[6] and (2) data from the files of the CCNSC detailing activity against the three tumors used in the CCNSC mouse tumor screening program—Sarcoma 180 in Swiss albino mice and Ca 755 mammary adenocarcinoma and L 1210 lymphatic leukemia in C 57/DBA hybrids.[5] It is to be emphasized that the criteria of positivity in this screen were in general more rigorous than in many of the published reports. Many of the compounds reported as positive would therefore have been adjudged negative in the CCNSC screen.

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* This paper was not presented at the conference on which this monograph is based; it is the final report of the Subcommittee on Tissue Culture and the Subcommittee on Microbiology, Screening Panel, Cancer Chemotherapy National Service Center, Bethesda, Md., and is included here as a valuable adjunct to the work already presented.

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These two categories of compounds are considered separately in the following analyses. They proved to be neither identical nor mutually exclusive. The necessary reservations with respect to the use of these data as a basis for comparison with either cytotoxicity or antimicrobial activity *in vitro* have been discussed in previous reports.[2, 4]

The methods used for determining cytotoxicity in mammalian cell culture[1, 2] and inhibitory activity in microbiological assay systems[4] have been described in detail and will not be reviewed here. The criteria used for the classification of a compound as "positive" in these *in vitro* systems were: a 50 per cent inhibitory dose (ID_{50}) at less than 1.0×10^{-4} gm./ml. in mammalian cell cultures,[2] and an ID_{50} at less than 1.0×10^{-3} gm./ml. in one or more of the 12 microbiological assay systems studied.[4] The reasons for the arbitrary choice of these concentrations as differential criteria for classification of a compound as positive and the effect of choosing activity at a different concentration as evidence of positivity on the results obtained are discussed elsewhere.[2, 4]

Results

Of the 180 compounds tested in at least 3 experimental tumor systems, 89 had been reported to exhibit antitumor activity in one or more of the systems studied.[5, 6] The proportions of these 89 compounds classified as positive by the arbitrary criteria used in the *in vitro* studies are indicated in TABLE 1. The sensitivity of individual microbiological assay systems varied from 39 per cent (*Candida albicans*) to 82 per cent (*Tetrahymena pyriformis*). The tumor-active compounds missed by the combined microbiological assay and the mammalian cell culture screens are summarized in TABLES 2 and 3, respectively. Of the 5 compounds missed by all 12 of the microbiological assays even at 1.0×10^{-3} gm./ml., mammalian cell cultures classified 2 as positive. Of the 20 compounds missed by mammalian cell cultures, the microbiological assays detected 17 at 1.0×10^{-3} gm./ml., and 11 at 1.0×10^{-4} gm./ml., but only 3 at 1.0×10^{-6} gm./ml. The 3 compounds missed by all *in vitro* systems were formamide (CCNSC X No. 20), *N*-methylacetamide (CCNSC X No. 44), and *N*-methylformamide (CCNSC X No. 188). In the microbiological systems, the frequency of "false positives" (that is, those compounds active *in vitro*, but with no reported antitumor activity) generally paralleled sensitivity. It

Table 1

Comparison of Microbiological Assays, Tissue Cell Cultures, and the CCNSC Mouse Tumor Screen in the Detection of Compounds with Reported Experimental Antitumor Activity

No.	*In vitro* test system*		Percentage classified as positive	
			Eighty-nine compounds reported as tumor active	Ninety-one compounds reported as tumor negative†
1	*Streptococcus faecalis*		67	43
2	*Lactobacillus casei*		73	40
3	*Escherichia coli*		67	46
4	*Lactobacillus arabinosus*		63	44
5	*Pediococcus cerevisiae*		54	36
6	*Lactobacillus fermenti*		60	42
7	*Candida albicans*		39	33
8	*Saccharomyces carlsbergensis*		80	54
9	*Streptococcus faecalis*		73	59
10	*Lactobacillus casei*		76	48
11	*Tetrahymena pyriformis*		82	56
12	*Neurospora crassa*		64	41
	Combined systems No. 1–12‡		95	63
t	Human tissue cell cultures§		79	21
	CCNSC *in vivo* mouse tumor screen			
	Sarcoma 180	(23/83)	28	None, by definition
	Ca 755	(27/78)	35	None, by definition
	L 1210	(14/82)	17	None, by definition
	Combined CCNSC tumors	(34/84)	40	None, by definition

* The criterion for classification of a compound as positive is 50 per cent inhibition (ID_{50}) at less than 1.0×10^{-3} gm./ml.[4]

† "False positives," so classified because of no reported antitumor activity.

‡ The criterion for classification of a compound as positive is an ID_{50} of less than 1.0×10^{-3} gm./ml. in *any* of the 12 systems.[4]

§ The criterion for classification of a compound as positive is an ID_{50} of less than 1.0×10^{-4} gm./ml.[2]

should be emphasized here, as previously,[2, 4] that this was not a random series of test compounds; hence, the indicated frequencies of false positives does not necessarily indicate the incidence that would be encountered in a truly random screening program.

As adjudged by these data, mammalian cell cultures appear to be superior to microbiological systems by virtue of a lower incidence of false positives. The sensitivity of 79 per cent compares favorably with the most sensitive (*Saccharomyces carlsbergensis*, 80 per cent; *Lactobacillus casei*, 76 per cent; and *T. pyriformis*, 82 per cent) microbiological systems (table 1). The combined microbiological results (that is, a positive result at 1.0×10^{-3} gm./ml. in any one of the 12 systems) detected a considerably greater proportion of the tumor-active compounds (95 per cent), but only at the expense of a significant increase in the frequency of false positives (table 1); while at 1.0×10^{-4} gm./ml., combinations of microbiological assay systems detected the same

TABLE 2

COMPOUNDS REPORTED TO BE TUMOR ACTIVE THAT WERE "MISSED" BY *IN VITRO* SCREENING PROCEDURES

CCNSC X No.	Chemical name	Classification in:	
		Microbiological systems	Tissue cell culture
20	Formamide	−	−
25	Colchicine	−	+
44	*N*-Methylacetamide	−	−
64	Strophanthin K	−	+
188	*N*-Methylformamide	−	−

TABLE 3

ACTIVITY IN MICROBIOLOGICAL ASSAYS OF COMPOUNDS REPORTED TO BE TUMOR ACTIVE THAT WERE NOT SIGNIFICANTLY CYTOTOXIC IN TISSUE CELL CULTURES

CCNSC X No.	Chemical name	Cytotoxicity in cell culture (ID$_{50}$ gm./ml.*) versus:		Micro-biological assay systems†	Reported antitumor activity *in vivo*[5]‡
		"Liver"	KB		
18	Isoriboflavin	10^{-4}–10^{-5}	$>10^{-4}$	11/12	2/4 (1±)
20	Formamide	$>10^{-4}$	$>10^{-4}$	0/12	3/4
39	Galactoflavin	10^{-4}–10^{-5}	$>10^{-4}$	11/12	2/3
41	Ethyl carbamate	$>10^{-4}$	$>10^{-4}$	2/12	21/29
44	*N*-Methylacetamide	$>10^{-4}$	$>10^{-4}$	0/12	6/16
68	AA-223 Lederle	10^{-4}–10^{-5}	$>10^{-4}$	10/12	3/4
113	Isopropyl *N*-phenylcarbamate	$>10^{-5}$¶	$>10^{-5}$¶	12/12	2/6
114	DL-Ethionine	$>10^{-4}$	$>10^{-4}$	3/12	5/22
134	2-Acetamido-1,3,4-thiadiazole	$>10^{-4}$	$>10^{-4}$	4/12	2/5
143	4-Amino-6-anilino-1,2-dihydro-2,2-dimethyl-*s*-triazine	10^{-4}–10^{-5}	$>10^{-4}$	4/12	3/8 (2±)
149	1,4-Dimethanesulfonyloxybutane (Myleran)	$>10^{-4}$	$>10^{-4}$	4/12	8/23
158	Hydrocortisone	10^{-4}–10^{-5}	$>10^{-4}$	7/12	8/15
188	*N*-Methylformamide	$>10^{-4}$	$>10^{-4}$	0/12	11/17
197	2-Ethylamino-1,3,4-thiadiazole hydrochloride	$>10^{-4}$	$>10^{-4}$	1/12	3/6
21	DL-Desthiobiotin§	$>10^{-4}$	$>10^{-4}$	2/12	1/4
95	Folic acid§	$>10^{-4}$	$>10^{-4}$	3/12	2±/14
150	Resorcinol§	$>10^{-4}$	$>10^{-4}$	3/12	1/3
151	β-2-Thienylalanine§	$>10^{-4}$	$>10^{-4}$	5/12	1/19
186	5,6-Diaminouracil hemisulfate§	$>10^{-4}$	$>10^{-4}$	11/12	1/6
198	Benzotriazole§	$>10^{-4}$	$>10^{-4}$	8/12	1,2±/6

* Concentration that caused 50 per cent inhibition of growth.[2]

† Proportion of systems inhibited at 1.0×10^{-3} gm./ml. Eight compounds (CCNSC X Nos. 20, 44, 188, 197, 21, 95, 150, 198) were negative at 1.0×10^{-4} gm./ml.; and only 3 compounds (CCNSC X Nos. 39, 68, 114) were positive at 1.0×10^{-6} gm./ml.[4]

‡ Proportion of tumor systems inhibited.

§ Compounds giving doubtful results in one or two tumor systems were arbitrarily included in this group.

¶ Could not be tested at higher concentration because of insolubility.

TABLE 4

ACTIVITY OF COMPOUNDS CLASSIFIED AS TUMOR ACTIVE BY CCNSC AND OTHER EXPERIMENTAL TUMOR SCREENS IN MAMMALIAN CELL CULTURES AND MICROBIOLOGICAL ASSAYS

In vitro screens[2,4,5] and criteria for activity	Percentage detected among compounds active in						False positives in "tumor-negative" series (91 compounds)
	CCNSC tumor screen (34 compounds)		Other tumor screens (55 compounds)		Total (89 compounds)		
	No.	Per cent	No.	Per cent	No.	Per cent	Per cent
Positive at 1.0×10^{-4} gm./ml.							
Human cell cultures	25	75	44	80	69	79	21
Microbiological assays*	28	82	41	75	69	79	29
Combination	31	88	45	82	78	86	36
Positive at 1.0×10^{-6} gm./ml.							
Human cell cultures	17	50	19	35	36	39	2.2
Microbiological assays*	14	42	13	24	27	31	2.2
Combination	19	56	23	43	42	48	4.4

* The three systems in combination I (TABLE 6).

number of tumor-active compounds as did mammalian cell cultures, but again with a higher rate of false positives (TABLE 4). It should be pointed out that many of the false-positive compounds so classified in mammalian cell cultures also were inhibitory in several of the microbiological assay systems (TABLE 5). As emphasized previously,[2,4] there is no means of determining whether it is the *in vitro* or the *in vivo* results that are erroneous in terms of activity in man.

The CCNSC 3-tumor mouse screen detected 40 per cent of the 84 purportedly tumor-active compounds examined.[5,7] It is to be noted that mammalian cell cultures alone, or combinations of as few as 3 microbiological assay systems, detected as many compounds as the CCNSC mouse tumor screen even when an ID_{50} at 1.0×10^{-6} gm./ml. or less was taken as the criterion of activity (TABLE 4). At this level of sensitivity, either mammalian cell cultures or a combination of microbiological assays contributed relatively few false positives. Of the 34 compounds detected by the CCNSC 3-tumor screen, 50 to 75 per cent were detected by the mammalian cell culture screen, and 42 to 82 per cent by the microbiological assay screens, the sensitivity in each case depending upon whether activity at 1.0×10^{-6} or 1.0×10^{-4} gm./ml. was used as the criterion for positivity. The combination of the 2 *in vitro* methods detected 56 to 88 per cent of the compounds classified as positive by the CCNSC 3-tumor screen (TABLE 4).

As pointed out previously,[4] although 12 microbiological assay systems were used in these studies, all were not necessary to attain the same result. A number of combinations of systems were as effective in the detection of tumor-active compounds as was the entire series. When used in conjunction with mammalian cell culture, for example, several 3-system combinations yielded the same results as were obtained with all 12 microbiological assays.

TABLE 5

COMPOUNDS REPORTED TO BE TUMOR NEGATIVE THAT WERE SIGNIFICANTLY CYTOTOXIC IN TISSUE CELL CULTURES AND MICROBIOLOGICAL ASSAY SYSTEMS

CCNSC X No.	Chemical name	Cytotoxicity in cell culture (ID_{50} gm./ml.*)	Microbiological assay systems†	Reported anti-tumor activity *in vivo*[5]‡
6	6-Methoxyquinoline-4-aldehyde	$>10^{-5}$ $<10^{-4}$	12/12	0/4
77	2,6-Dimethyl-4-(2-oxo-2-phenylethylmercapto)pyrylium bromide	$>10^{-5}$ $<10^{-4}$	4/12	0/3
89	Chloramphenicol (Chloromycetin)	$>10^{-5}$ $<10^{-4}$	9/12	0/20
94	2,4′-Sulfonyldiphenol	$>10^{-5}$ $<10^{-4}$	11/12	0/3
100	Methyl chloracetate	$>10^{-5}$ $<10^{-4}$	9/12	0/3
111	8-Azaxanthine	$>10^{-5}$ $<10^{-4}$	10/12	0/23
121	Ethylhydrocupreine hydrochloride	$>10^{-5}$ $<10^{-4}$	10/12	0/3
126	4-Amino-6-(*p*-chloroanilino)-1,2-dihydro-2,2-dimethyl-*s*-triazine·1 H_2O	$>10^{-5}$ $<10^{-4}$	4/12	0/4
131	*p*-Chlorophenol	$>10^{-5}$ $<10^{-4}$	12/12	0/3
140	Cotarnine hydrochloride	$>10^{-5}$ $<10^{-4}$	2/12	0/5
145	Thebaine hydrochloride (ethanolate)	$>10^{-5}$ $<10^{-4}$	3/12	0/4
192	Cinchonidine	$>10^{-5}$ $<10^{-4}$	11/12	0/3
11	*p*-Anisaldehyde thiosemicarbazone	$>10^{-6}$ $<10^{-5}$	11/12	0/3
35	α-Chloroacetanilide	$>10^{-6}$ $<10^{-5}$	10/12	0/4
55	*n*-Lauryl thiocyanate	$>10^{-7}$ $<10^{-6}$	9/12	0/4
67	Di-*n*-octylamine	$>10^{-6}$ $<10^{-5}$	12/12	0/3
98	4-Chlorochalcone	$>10^{-6}$ $<10^{-5}$	10/12	0/3
82	Narcotine	$>10^{-6}$ $<10^{-5}$	11/12	0/5
103	Pyronin B	$>10^{-7}$ $<10^{-6}$	12/12	0/6

* Concentration that causes 50 per cent inhibition of growth. Where results with the two cell lines differed, that of the more sensitive cell is listed.[2]

† Proportion of systems inhibited at 1.0 × 10^{-3} gm./ml. All but 3 compounds (CCNSC X Nos. 126, 140, 145) also were positive at 1.0 × 10^{-4} gm./ml.; and 3 compounds (CCNSC X Nos. 89, 103, 111) were positive at 1.0 × 10^{-6} gm./ml.[4]

‡ Proportion of tumor systems inhibited.

The use of microbiological assay systems results in a considerable increase in the frequency of false positives (TABLE 6). However, of the 54 compounds classified as positive at 1.0 × 10^{-3} gm./ml. by microbiological assay systems, over and above those so classified by mammalian cell cultures, 17 (31 per cent) are reported to exhibit experimental antitumor activity (TABLE 6).

TABLE 6

SENSITIVITY AND RELIABILITY OF HUMAN CELL CULTURES AND MICROBIOLOGICAL ASSAYS IN DETECTION OF TUMOR-ACTIVE COMPOUNDS

	Eighty-nine tumor-active compounds[2, 4, 5]		Ninety-one tumor-negative compounds[2, 4, 5]	
	No. compounds classified as positive when tested at:			
Human cell cultures	1.0×10^{-4} gm./ml.	1.0×10^{-6} gm./ml.	1.0×10^{-4} gm./ml.	1.0×10^{-6} gm./ml.
Microbiological assays	1.0×10^{-3} gm./ml.	1.0×10^{-6} gm./ml.	1.0×10^{-3} gm./ml.	1.0×10^{-6} gm./ml.
I. Human cell cultures	69	35	20	2
Lactobacillus casei No. 10	9	1	25	2
Lactobacillus casei No. 2	3 }17*	4 }7	4 }37	0 }2
Neurospora crassa No. 12	5	2	8	0
Combination	86 (97%)	42 (48%)	57 (62%)	4 (4.4%)
II. Human cell cultures	69	35	20	2
Saccharomyces carlsbergensis No. 8	10	3	34	2
Lactobacillus casei No. 10	4 }17	1 }6	1 }39	1 }4
Neurospora crassa No. 12	3	2	4	1
Combination	86 (97%)	41 (46%)	59 (65%)	6 (6.6%)
III. Human cell cultures	69	35	20	2
Tetrahymena pyriformis No. 11	12	0	32	0
Lactobacillus casei No. 2	3 }17	4 }6	3 }40	0
Neurospora crassa No. 12	2	2	5	0
Combination	86 (97%)	41 (46%)	60 (66%)	2 (2.2%)

* Numbers in brackets represent compounds classified as positive by each system over and above those so classified by human cell cultures and excluding those detected by another microorganism in the same group.

Discussion

The inherent premise in any screening program is that it will select agents that ultimately will prove to exhibit that biological property which the screening device was designed to select. In the detection of antibacterial agents, even when the human infection can be approximated in experimental animals, the development and application of reliable screening procedures has presented difficulties. These difficulties are compounded when the screen is designed for the recognition of agents that may be useful in the therapy of neoplastic disease in man. The varieties of the human disease, each with its own biological idiosyncrasies, and the important dissimilarities between human neoplasia and experimental tumors, are obvious complications.

In any screening procedure the major problem is one of delineating reasonably satisfactory parameters of sensitivity and reliability. The absence of a fixed point of reference makes this particularly difficult in the case of a screen for antitumor activity. For the present, any new screening device must be compared with activity in experimental tumor systems, this despite the known lack of correlation between activity in experimental tumor systems and in human neoplasia. Interscreen comparisons may have no bearing on the ultimate usefulness of the compounds in the chemotherapy of the human disease. Thus, the criteria for positivity in the CCNSC mouse tumor screen were purposely set at such a level that a number of compounds previously reported to be tumor active were rejected. The compounds selected as positive by the CCNSC tumor screen are necessarily those that are highly active for the particular tumors used. Those compounds may, however, be no more useful in the chemotherapy of human tumors than other compounds not quite sufficiently active to be characterized as positive in the CCNSC screen, or that exhibit activity either in different experimental tumors or in other assay systems.*

With respect to the sensitivity of the cell culture and microbiological assay screens, the data previously presented,[1, 2, 4] as well as the over-all comparison here discussed, indicate that these relatively simple, rapid, and inexpensive *in vitro* assays, used either alone or in combination, will detect the majority of those agents known to exhibit experimental antitumor activity (TABLES 4 and 6). Eighteen to 20 experimental tumor systems would be required to detect all of the compounds classified as positive by a single mammalian cell culture assay of cytotoxicity.[4]

As to reliability, either *in vitro* method classifies as positive a number of compounds that are negative in experimental tumor assays (TABLE 5). By definition, in the framework of the present comparison these are false positives, but their true classification in terms of the ultimate criterion, activity in man, is not clear. Conversely, the true classification of compounds positive by experimental tumor assay and inactive *in vitro* (TABLES 2 and 3) is equally uncertain.†

From this and previous studies it is evident that mammalian cell cultures and microbiological assays may provide a new approach to the screening of compounds for antitumor activity. Either or both methods are suitable for routine large-scale use, with considerable economy of time and effort, as compared with primary screening in experimental tumors. That these *in vitro* methods will not eliminate the use of animal tumors is evident. However, it is possible that the best use of experimental tumor systems may lie in the secondary and detailed study of those active compounds turned up by the simpler and more rapid primary *in vitro* screens.

Summary

Previous reports have indicated that the majority of compounds exhibiting antitumor activity also are significantly cytotoxic in mammalian cell cultures, and inhibitory in microbiological assay systems. A combination of a mam-

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* By one set of criteria and in certain tumors, 4 aminopteroylglutamic acid (Aminopterin) was adjudged negative. By other criteria, in other experimental tumor systems and in man as well, it has definite antitumor activity.

† The discrepancy between the results obtained in experimental tumors and in man underlines the need for newer and more satisfactory screening procedures. Human tumors growing in the hamster cheek pouch[8] may prove to be a better analogue of human neoplasia than transplanted animal tumors.

malian cell culture (at 10^{-4} gm./ml.) and 3 microbiological assay systems (at 10^{-3} gm./ml.) classified as positive 97 per cent of the 89 reference compounds reported to have antitumor activity. Of the 34 compounds in this group classified as positive by the CCNSC mouse tumor screen, the combined *in vitro* assays classified 88 per cent as positive. At 1.0×10^{-4} gm./ml. the human cell culture screen gave 21 per cent false-positive results in 91 tumor-negative compounds, and the microbiological assays gave 29 per cent false-positive reactions.

When the compounds were tested at 10^{-6} gm./ml., the combined *in vitro* assay detected 48 per cent of all the tumor-active compounds and 56 per cent of the CCNSC-positive compounds, with 4.4 per cent false positives.

The problems inherent in interscreen comparisons, and the difficulty of assessing the significance of their sensitivity and reliability in terms of the human disease, are discussed. Both *in vitro* methods contributed false-positive and false-negative results; however, since the same situation obtains with the experimental tumor assays used as a reference base, this does not necessarily invalidate the *in vitro* procedures.

These studies indicate the need for large-scale studies to determine the usefulness of mammalian cell cultures and microbiological assay systems in the primary screening of candidate antitumor agents.

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SUMMARY OF THE MONOGRAPH

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We have been fortunate in having some differences of opinion expressed here, but I am confident that we can all agree in our appreciation of this publication. It is my purpose here to separate some of the main points from the mass of detail presented. Any consideration of chemotherapy studies may be divided into the interdependent aspects, the test objects, the test materials, the test procedures, and the evaluation of the results. In this monograph an effort has been made to minimize the presentation of new chemotherapeutic agents in order to place emphasis upon the screening procedures. In this the contributors succeeded well and presented results for the most part upon known agents, the data being provided to aid in the evaluation of the respective test methods. In a few instances the procedures were ones designed to detect a restricted group of agents, for example, Hitchings' studies with *Lactobacillus caseii* to detect antimetabolites of purines and pyrimidines. Other procedures with a circumscribed area of usefulness employ test tumors responsive to hormone therapy.

Another aspect of the reports for which we are indebted is the enthusiasm with which the various test objects and procedures have been presented, an enthusiasm sufficient to interest others and, at the same time, marked by a conservatism in recognition of the considerable amount of work remaining to establish the value of any new procedure. The great problems confronting us were acknowledged with the oft-repeated complaint that we do not yet have yardsticks adequate in number to measure any new procedure. We do not have compounds sufficiently active against cancer in man. We still know too little about so-called negative compounds.

This publication was designed as an examination of new test objects and new procedures and as an evaluation of old techniques. To accomplish this, the presentations have progressed from the simpler test objects, the microorganisms, through tissue culture and ascites cells to more complex systems of lymphomas, hormone-responsive tumors, various special systems, and the human tumor in the heterologous host. The latter has been considered attractive as possibly providing the test system nearest our ultimate test objective: cancer in man. The presentations of nearly all the workers with the human tumors, and that of Palm in particular, have made clear that, although the system is most attractive theoretically, it does present numerous practical problems. It is encouraging to see that some of the problems that previously have seemed to be handicaps have been overcome satisfactorily. The developments leading to the possible use of the mouse as a heterologous host for the human tumor may be one of the most important of the recent developments. Woolley has provided a useful summary of the present status of the heterologously grown human tumor as a test object for screening studies.

Several of the contributors have provided most helpful backgrounds for the various sections. For example, Toolan, Dunn, Furth, Segaloff, Moore, and Leighton, in providing information about such test objects as the human tumor in the heterologous host, the mouse lymphosarcomas, hormone-responsive tumors, and cells in tissue culture, have indicated the potential usefulness of the test objects as well as some of the problems likely to be encountered in their use.

Careful study by the microbiology and tissue culture groups have provided a large amount of data. An attempt to compare these with experimental animal tumor data revealed shortcomings in the information available in the literature (see APPENDIX). Therefore it is necessary to enter a plea for more animal tumor data as well as for the human cancer data.

Microorganisms, so easily handled, have been presented as possible aids to the problems of the multicellular forms of life. Schabel has discussed the use of resistant microorganisms, which may provide a useful follow-up for the screening results and may show something of the mechanism of action. Differences in resistance patterns must be taken into consideration, as pointed out by Hutchison, and they may be found to provide useful information beyond that of a single resistant mutant. Hutner advocated the use of humanoid microorganisms. It must be recognized that, no matter how theoretically different test systems may be from the objective, human cancer, still there may be a role for the special system. Szybalski presented a special microbiological assay involving mutagenesis which, if its application would save only 10 to 15 per cent of the tests in experimental animals, would mean large savings in time, space, and finances in the work with mice, a desirable end when one considers that millions of dollars are now going into screening. Bradner has presented indications of possible savings to be achieved by the use of an anaerobic microorganism test that might suggest appropriate dose levels for use in mice.

Tissue culture investigators have been concerned with the question of what normal cells are the appropriate controls in tissue culture and whether any cell in tissue culture truly can be called normal. Gey has emphasized the problem of variability in cell lines; this is a problem that must be recognized in screening programs—the variation in characteristics that may be encountered in the test object. Such variations in tumors, regardless of the selection pressure, raise the question of tumor nomenclature. In different laboratories with different degrees of sensitivity to chemotherapeutic agents, can Sarcoma 180 be called merely Sarcoma 180? Can Sarcoma 180 grown in the mouse from cells carried in tissue culture be left without a special designation to distinguish it from the mouse-to-mouse passage material? Should not a line of human tumor grown in the hamster be distinguished in some way from the same human tumor that has been carried in the rat? Adoption of a system of nomenclature as has been done for the various lines of tumors or leukemias with different resistances to chemotherapeutic agents may be necessary to prevent a chaotic condition when attempts are made to compare the results of screening or of biochemical studies.

Sassenrath's report of differences in responses among a number of ascitic tumors tested with selected agents has dimmed hopes that use might be made of the relative sensitivity of ascitic tumors to provide a simple test that would detect nearly all antitumor substances, although probably at the expense of many false positives. Use of certain ascites tumors for the special objective of screening compounds for effectiveness against metastatic growth was presented by Yoshida. Some of his rat tumors may provide suitable tools by which to screen for suitable adjuncts of surgery.

Among the reports on the lymphomas, two in particular represent contributions that should initiate interesting developments. One is the intriguing news of Fischer's cultivation of leukemia cells in tissue culture. The other is the description and use of Dunning's leukemia for screening purposes. The initial results of tests with this leukemia, as reported by Dunning and by Jones, point to its probable utility.

Various speakers have emphasized the need for tumors with hormone responsiveness adequate to be suitable for screening steroids and other hormones for antitumor activity. It is encouraging that Furth is starting to investigate this problem with the tumors he has developed.

Reports on the heterologously grown human tumors indicate that much of the spade work has been done and, while problems remain, systems are in use with defined responses to standard agents. With this foundation it will be of interest to see whether these systems will justify their use.

A miscellaneous group of test systems was covered in Part VII. Included were such diverse test objects as resistant leukemias, avian virus tumors, tumors in dogs, granulomas, spontaneous and induced tumors in the rat, and an *in vitro* test with ascitic tumor cells. As Shear, the chairman of that section, aptly commented, these were labeled as special systems merely because they are not commonly used. Adequate study of them might well reveal characteristics demanding consideration of the test objects for "standard" systems. Also included in this section was a report on "The Role of Chance Observations in Chemotherapy." This was included, as Shear correctly assumed, to emphasize the fact that, while we know the advantages of systematic work, we should not be blinded to the possibility that accidental observations made in other directions may provide leads to this field quite as useful as those gained by systematic and painstaking work.

In his introductory comments, Litchfield pointed out that some degree of success in screening must have been achieved, for he noted that combination therapy was a topic for discussion. This was discussed with somewhat divergent views on studies for combination effects, which to some extent were reconciled by Goldin in his discussion. It is clear that one must define his objectives in any screening program; thus, one protocol will be better suited to screen for effective combinations while another will be more useful for the detailed mapping of contours in the effectiveness of a multiplicity of ratios of paired components of a particular combination. It is a problem for the statisticians to determine a compromise plan of tests that would cut to a minimum the detailed mapping with a maximum opportunity of revealing the nearly optimum ratio for a combination. Although the simpler protocol discussed by Clarke revealed the optimum, or nearly the optimum, combination of 6-mercaptopurine and azaserine selected by the more elaborate protocol presented by Mantel, it is not clear how frequently the simpler protocol would be as effective with different combinations.

We must agree with Lasagna and others that we cannot be unduly wedded to a particular screening object or technique. Neither should we be too hasty in discarding any of them. It has been suggested by one of the discussants that failure in man of an agent curative of a cancer in the animal would justify dropping that particular experimental tumor. I should hate to see any test object or procedure discarded on the basis of a single failure. We should seek the precise reason for the failure. Furthermore, it can be anticipated that various agents may be curative in animal cancers because immune mechanisms come into play to complete the destruction of the tumor damaged initially by the chemotherapeutic agent. While it may be hoped that immune mechanisms will lend themselves to a controlled role in human cancer, it is not expected that we shall see as great an activity against an individual's own cancer as may be seen in the animal with transplanted tumors. There is no reason to decry the assistance that immune mechanisms in the experimental animal give us in detecting a limited amount of damage from chemotherapeutic agents. This is, I believe, a form of combination therapy making the experimental animal tumors more sensitive and more useful test objects.

The reports included in this monograph have been presented with an objectivity that, if continued in the studies in progress, should lead to the development of better procedures and, ultimately, to the chemotherapeutic agents we seek.

APPENDIX

SUMMARY OF RESULTS OF ANTITUMOR TESTS ON 200 SELECTED COMPOUNDS

NSC No.	Compound name	CCNSC screening results*			Other screening data†
		S 180	Ca 755	L 1210	
4	2-Amino-5-nitrothiazole	− (300)‡	− (100)	− (300)	0/0
145	Phenylphosphonic acid	− (500)	− (125)	− (125)	0/0
185	3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide (Actidione)	+ (32)	− (16)	± (32)	3/5
198	3-Hydroxy-2-methyl-3-(2-pyridyl)-propiophenone	− (500)	− (250)	− (500)	0/0
280	9-(3-Dibutylaminopropylamino)-1,2,3,4-tetrahydroacridine phosphate	− (50)	− (25)	− (35)	0/0
298	α-Cyanocinnamic acid	− (100)	− (35)	− (100)	0/0
311	2-Chloroacetanilide	− (175)	− (87.5)	− (87.5)	0/0
339	1,4-Dinitrosopiperazine	− (75)	− (40)	− (60)	0/1
350	(α,α-Dimethylbenzyl)trimethylammonium iodide	− (25)	− (7.0)	− (20)	0/0
355	7-Chloro-4-(4-diethylamino-1-methylbutylamino)-3,6-dimethylquinoline diphosphate dihydrate	− (250)	− (54)	− (200)	0/0
423	2-(2,4-Dichlorophenoxy)ethanol	− (125)	− (67.5)	− (125)	0/0
429	1,3-Diethylurea	− (500)	− (250)	− (500)	0/0
437	3-Mercaptopropionic acid	− (4.0)	− (2.0)	− (4.0)	0/0
441	2-Amino-2-methyl-*l*-propanol	− (125)	− (62.5)	− (125)	0/0
449	6-Methoxycinchoninaldehyde	− (175)	− (87.5)	− (175)	0/0
463	Brucine sulfate	− (100)	− (50)	− (100)	0/0
552	3-Methoxypropylamine	− (100)	− (50)	− (100)	0/0
553	*N*-(Hydroxymethyl)acrylamide	− (350)	− (175)	− (350)	0/0
579	*p*-Fluoroaniline	− (250)	− (85)	− (87.5)	0/0
643	Benzenesulfonic acid hydrazide	− (125)	− (37.5)	− (125)	0/1
712	*p*-Anisaldehyde thiosemicarbazone	− (175)	− (87.5)	− (175)	0/1
739	*N*-(*p*-[(2,4-Diamino-6-pteridylmethyl)amino]benzoyl)glutamic acid (Aminopterin)	− (0.01)	− (0.01)	− (0.01)	11/15
740	*N*-(*p*-[(2,4-Diamino-6-pteridylmethyl)methylamino]benzoyl)-glutamic acid (Methotrexate)	+ (0.5)	+ (0.5)	+ (3.0)	11/21
741E	17-Hydroxycorticosterone, 21-acetate	+ (35)	+ (37.5)	− (37.5)	8/14
742	L-Serine diazoacetate (azaserine)	+ (5.0)	+ (4.0)	+ (5.0)	12/21
743	2,6-Diaminopurine hydrate	− (90)	− (90)	− (90)	5/28
744	6-Chloropurine	− (30)	+ (100)	− (30)	1/1
746	Carbamic acid, ethyl ester	+ (250)	+ (250)	− (750)	19/28
747	*N*-Methylacetamide	− (1500)	− (650)	− (300)	6/15
748	Formamide	+ (250)	+ (250)	± (250)	2/3
749	5-Amino-1H-*v*-triazolo-[*d*]-pyrimidin-7-ol (8-azaguanine)	+ (100)	+ (50)	+ (75)	18/41
750	Methanesulfonic acid, tetramethylene ester (Myleran)	+ (30)	± (15)	− (30)	7/21

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* Symbols: − = no significant tumor inhibition or increase in survival time (for L 1210); ± = significant tumor inhibition (or increase in survival time) in one trial; + = significant tumor inhibition (or increase in survival time) in at least two trials.

† Literature survey (by Dean Gamble, CCNSC) of animal tumor reports other than CCNSC tests. The numerator indicates the number of "positive" reports. The denominator indicates the number of reports in the literature survey.

‡ Number in parentheses indicates the highest nontoxic dose tested.

APPENDIX—*Continued*

NSC No.	Compound name	CCNSC screening results			Other screening data
		S 180	Ca 755	L 1210	
751	DL-2-Amino-4-(ethylthio)butyric acid	− (100)	− (75)	− (75)	5/22
752	2-Amino-6-purinethiol	+ (50)	+ (5)	± (5)	1/1
753	Purine	+ (500)	− (180)	− (200)	2/4
754	2-Thiophenalanine	− (750)	− (750)	− (400)	1/17
755	6-Purinethiol hydrate	+ (50)	+ (15)	+ (20)	30/41
756	1H-*v*-Triazolo-[*d*]-pyrimidine-5,7-diol (8-azaxanthine)	− (750)	− (750)	− (250)	0/22
757	Colchicine	− (0.05)	+ (0.2)	− (0.5)	24/51
758	D-Glucosamine hydrochloride	− (1500)	− (1500)	− (750)	1/18
759	Benzimidazole	− (250)	− (250)	− (200)	1/21
761	Methyl 3-pyridyl ketone	− (250)	− (135)	− (250)	0/3
762	2,2′-Dichloro-*N*-methyldiethylamine hydrochloride	+ (1.5)	+ (0.7)	− (1.5)	27/40
763	Methyl sulfoxide	− (500)	− (500)	− (500)	0/0
805	Meconic acid	− (300)	− (50)	− (300)	0/0
824	Camphoric acid	− (500)	− (125)	− (400)	0/0
886	3,5-Dibromoanthranilic acid	− (500)	− (250)	− (500)	0/0
1112	Cyclopropanecarboxylic acid	− (125)	− (60)	− (125)	0/0
1136	Lauric acid hydrazide	− (60)	− (30)	− (60)	0/0
1195	Thiocyanic acid, 2,2′-oxydiethylene ester	− (7)	− (1)	− (7)	0/2
1228	3-(6-Chloro-4-methyl-2-quinazolinyl)-1,1-dimethylguanidine nitrate hydrate	− (90)	− (45)	− (90)	0/0
1256	Lactic acid, allyl ester	− (250)	− (125)	− (63)	0/0
1355	1,3:2,4-Di-*O*-benzylidene-DL-ribitol	− (125)	− (60)	− (125)	0/0
1478	Thiocyanic acid, butyl ester	− (25)	− (9.0)	− (7.5)	0/0
1481	Thiocyanic acid, dodecyl ester	− (100)	− (50)	− (50)	0/0
1532	2,4-Dinitrophenol	− (25)	− (22.5)	− (25)	1/5
1571	Resorcinol	− (500)	− (250)	− (500)	0/1
1578	3-Pyridinesulfonic acid, sodium salt	− (500)	− (250)	− (500)	0/3
1589	1,2,3,4-Tetrahydro-2,4-dioxo-5-pyrimidinecarboxylic acid monohydrate	− (62.5)	− (56.25)	− (62.5)	0/2
1590	4′-Formylacetanilide semicarbazone	− (31)		− (31)	0/0
1765	Dioctylamine	− (25)	− (13)	− (50)	0/1
1872	Picryl chloride	− (5)	− (2.5)	− (5)	0/0
1873	4′-Amino-*o*-acetanisidine	− (500)	− (150)	− (500)	0/0
1896	2-Methylpseudourea hydrochloride	− (500)	− (250)	− (250)	0/1
1901	Glycocyamine	− (500)	− (250)		0/4
1903	Acetylenedicarboxylic acid	− (60)	− (18)	− (30)	0/1
1911	Thiocyanic acid, ethylene ester	− (20)	− (10)	− (20)	0/1
1912	2-Aminopyrimidine	− (500)	− (250)	− (500)	0/6
1921	2,6-Diaminopyridine	− (50)	− (25)	− (13)	0/2
2012	1-Dodecyl-4-nonylpyridinium chloride			− (.05)	0/0
2030	4,6-Dimethyl-2-pyrimidinol hydrochloride	− (500)	− (250)	− (500)	0/0
2039	8-Quinolinol	− (60)	− (35)	− (60)	0/3
2059	2-Chlorotriethylamine hydrochloride	− (18)	− (9)	− (18)	0/1
2066	Theophylline	− (250)	− (125)	− (250)	0/1
2074	Heptylamine	− (125)	− (18)	− (60)	0/1
2092	3-Benzoylpropionic acid	− (250)	− (200)	− (250)	0/2
2100	5-Nitro-2-furaldehyde semicarbazone (nitrofurazone)	− (175)	− (60)	− (125)	1/5
2105	Carbanilic acid, isopropyl ester	− (150)	− (75)	− (150)	2/4
2107	1-(5-Nitrofurfurylideneamino)hydantoin (Furadantin)	− (125)	− (21.6)	− (25)	0/2

Appendix—*Continued*

NSC No.	Compound name	CCNSC screening results			Other screening data
		S 180	Ca 755	L 1210	
2183	3-Chloropropionic acid	− (250)	− (125)	− (250)	0/0
2261	2-(2-Methoxyethoxy)ethanol	− (500)	− (250)	− (500)	0/0
D 2359	*N*-Phenylsuccinimide	− (333)	− (117)	− (333)	0/0
2432	2,4′-Sulfonyldiphenol	− (250)	− (250)	− (500)	0/0
2515	2,6-Dimethyl-4-(phenacylthio)-pyrylium bromide	− (125)	− (60)	− (63)	0/0
2550	Tetrapropyl thiopyrophosphate	− (250)	− (225)	− (250)	0/1
2635	Chloroacetic acid, methyl ester	− (25)	− (12.5)	− (25)	0/0
2639	Trichloroacrylic acid, sodium	− (500)	− (250)	− (500)	1/2
2643	4-Chlorochalcone	− (500)	− (250)	− (500)	0/2
2652	2-Dimethylaminoethanol	− (500)	− (250)	− (500)	0/0
2657	Carbonic acid, allyl *p*-chlorophenyl ester	− (300)	− (150)	− (150)	0/0
2671	Ethylphosphonic acid, diethyl ester	− (500)	− (250)	− (500)	0/2
2675	Butyl phosphite	− (500)	− (250)	− (500)	0/0
2764	4-Acetylmorpholine	− (500)	− (250)	− (500)	0/3
2773	*N*-2-Hydroxyethylphthalimide	− (150)	− (150)	− (150)	0/0
2789	5-Iodosalicylic acid	− (500)	− (250)	− (125)	0/1
2852	3-Hydroxy-7-sulfo-2-naphthoic acid	− (500)	− (250)	− (500)	0/0
2853	*m*-Sulfobenzoic acid	− (250)	− (125)	− (250)	0/0
2857	Diethylene glycol dibenzenesulfonate	− (300)	− (300)	− (300)	0/0
2859	3-(Methylthio)-1-propanol	− (500)	− (250)	− (250)	0/0
2877	*p*-Chlorophenol	− (55)	− (40)	− (55)	0/0
2879	2,4-Dichlorophenol	− (250)	− (87)	− (63)	0/0
2897	2,2′-Sulfinyldiethanol	− (500)	− (250)	− (500)	0/1
2902	*p*-Nitrobenzamide	− (300)	− (100)	− (300)	0/3
3051	*N*-Methylformamide	+ (175)	+ (175)	− (200)	12/21
3052	Methanesulfonic acid, nonamethylene ester	− (15)	− (13.5)	± (15)	18/23
3053	Actinomycin D	− (.025)	± (.0225)	− (0.025)	3/27
3054	Carbamic acid, methyl ester	− (750)	− (750)	− (300)	0/2
3055	Puromycin dihydrochloride	− (100)	+ (90)	− (100)	2/4
3056	9-(3-Amino-3-deoxy-β-D-ribofuranosyl)-6-dimethylamino-β-purine (puromycin aminonucleoside)	± (50)	+ (45)	+ (50)	3/3
3057	5,7-Diamino-1H-*v*-triazolo-[*d*]-pyrimidine sulfate (2,6-diamino-8-azapurine sulfate)	− (40)	− (36)	− (40)	0/1
3058	1H-Benzotriazole	− (250)	+ (225)	− (250)	0/3
3059	5-Chlorobenzimidazole	− (250)	− (125)	− (250)	0/1
3060	Potassium arsenite	± (15)	± (13)	− (8)	7/23
3061	2,4-Diamino-5-(*p*-chlorophenyl)-6-ethylpyrimidine (Daraprim, pyrimethamine)	− (125)	+ (100)	− (40)	3/14
3062	2,4-Diamino-5-(3,4-dichlorophenyl)-6-ethylpyrimidine	− (12)	+ (6)	− (12)	10/15
3063	5-Hydroxy-4,6-dimethyl-3-pyridinemethanol hydrochloride (deoxypyridoxine hydrochloride)	+ (225)	− (225)	− (50)	2/19
3064	10-(2-Hydroxyethyl)-7,8-dimethylisoalloxazine acetate	− (125)	− (115)	− (125)	0/0
3065	10-(2-Hydroxyethyl)-7,8-dimethylisoalloxazine	+ (500)	+ (450)	− (500)	1/2
3066	10-(2-Hydroxyethyl)-7,8-dimethylisoalloxazine succinate, sodium salt	− (200)	− (180)	− (200)	0/0
3067	Netropsin hydrochloride	− (20)	− (3.1)	− (20)	4/16
3068	6-Nitrobenzimidazole	− (200)	− (200)	− (200)	0/1

Appendix—*Continued*

NSC No.	Compound name	CCNSC screening results			Other screening data
		S 180	Ca 755	L 1210	
3069	Chloramphenicol (Chloromycetin)	− (250)	− (1000)	− (250)	0/18
3072	Sodium azide	− (9)	− (8)	− (9)	0/1
3073	Folic acid	− (50)	− (45)	− (50)	2/12
3074	4,6-Diamino-1-(*p*-chlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride	± (50)	− (30)	− (50)	0/1
3075	*p*-(4,6-Diamino-2,2-dimethyl-*s*-triazin-1(2H)-yl)benzoic acid hydrochloride	− (25)	− (22)	− (25)	0/0
3076	4-Amino-6-anilino-1,2-dihydro-2-phenyl-*s*-triazine	− (25)	− (20)	− (25)	0/0
3077	4,6-Diamino-1-(3,4-dichlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride	− (6)		− (2.5)	0/1
3080	4,6-Diamino-1-(*m*-chlorophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride	− (25)	+ (25)	+ (25)	0/0
3081	4,6-Diamino-1-(*p*-chlorophenyl)-2-hexyl-1,2-dihydro-*s*-triazine hydrochloride	− (25)	− (20)	− (25)	0/0
3082	4,6-Diamino-1-(*m*-bromophenyl)-1,2-dihydro-2,2-dimethyl-*s*-triazine hydrochloride	− (25)	+ (25)	± (25)	0/0
3083	4,6-Diamino-1-(*m*-chlorophenyl)-2-hexyl-1,2-dihydro-*s*-triazine hydrochloride	− (25)	− (22)	− (25)	0/0
3084	2-Amino-4-(methylsulfinyl)butyric acid	− (150)	− (135)	− (150)	0/3
3085	DL-5-Methyl-2-oxo-4-imidazolidinehexanoic acid (DL-dethiobiotin)	− (100)	− (90)	− (100)	1/4
3086	*N*-(2,4-Dihydroxy-3,3-dimethylbutyryl)taurine (pantoyltaurine)	− (200)	− (180)	− (200)	0/1
3087	2-Amino-4-arsenosophenol hydrochloride (oxophenarsine hydrochloride)	− (10)	− (9)	− (10)	1/1
3088	4-(*p*-[*N*,*N*-Bis(2-chloroethyl)amino]phenyl)butyric acid (CB 1348)				
3089	Methylene blue (methylthionine chloride)	− (50)	− (50)	− (50)	2/10
3090	Crystal violet (gentian violet)	− (2.5)	− (2.0)	− (2.5)	2/3
3091	Methyl green	− (10)	− (9)	− (10)	1/3
3092	Neutral red	− (50)	− (45)	− (50)	1/7
3093	Pyronine B	− (10)	− (9)	− (10)	0/0
3094	3,3-Dimethyl-1-phenyltriazene	+ (65)	− (40)	+ (65)	13/20
3095	1,5-Diaminobiuret	± (150)	+ (75)	− (87.5)	3/14
3096	Deacetyl-*N*-methylcolchicine (demecolcine)				0/1
3097	3,3′-Diamino-4,4′-dihydroxyarsenobenzene dihydrochloride (arsphenamine, Ehrlich 606, Salvarsan)	± (100)	± (90)	− (100)	2/13
3098	5,7-Dihydroxy-[1,2,3]oxadiazolo-[5,4-*d*]pyrimidine	− (85)	− (75)	− (85)	0/4
3099	10-*d*-Dulcityl-7,8-dimethylisoalloxazine (galactoflavin)	± (500)	+ (200)	− (500)	0/1
3100	Isoriboflavin	− (85)	− (75)	− (85)	0/0
3102	5,6-Diaminouracil sulfate	− (175)	− (90)	− (175)	1/3
3115	Salicylamide	− (250)	− (70)	− (250)	0/1

APPENDIX—*Continued*

NSC No.	Compound name	CCBSC screening results			Other screening data
		S 180	Ca 755	L 1210	
3121	2,4-Dimethyl-3-pentanone semicarbazone	− (250)	− (125)	− (250)	0/1
3123	Amidinourea sulfate	− (500)	− (250)	− (500)	0/1
3141	1,2,3,6-Tetrahydro-3,6-dioxo-2-phenyl-4-pyridazineacetic acid	− (500)	− (250)	− (500)	0/3
3143	*N*-(2-carboxyethyl)-L-tyrosine	− (500)	− (250)	− (500)	0/1
3181	Lauranilide	− (500)	− (250)	− (500)	0/0
3206	2-Benzyl-2-thiopseudourea hydrochloride	− (60)	− (30)	− (60)	0/1
3210	Chloral	− (500)	− (250)	− (500)	0/1
3212	Chloromethyl *p*-chlorophenyl sulfone	− (300)	− (150)	− (300)	0/1
3225	Ethylene sulfite (cyclic)	− (500)	− (250)	− (500)	0/1
4236	4-(*p*-Dimethylaminostyryl)quinoline	− (40)	− (40)	− (36)	5/5
4237	4-(*p*-Dimethylaminostyryl)-1-methylquinolinium chloride	− (1.6)	− (1.0)	− (0.4)	2/5
4238	2-(*p*-Dimethylaminostyryl)-1-methylquinolinium iodide	− (4)	− (2.8)	− (4.0)	0/3
4239	4-(*p*-Dimethylaminostyryl)-1-methylquinolinium iodide	− (0.8)		− (0.8)	5/7
4240	4-(*p*-Diethylaminostyryl)quinoline	− (50)	− (15)	− (50)	4/4
4241	4-(*p*-Diethylaminostyryl)quinoline monohydrochloride	+ (250)	+ (200)	− (250)	1/4
4242	4-(*p*-Diethylaminostyryl)quinoline dihydrochloride	− (40)	+ (40)	− (40)	1/1
4320	Strophanthin K	− (5)	− (4)	− (5)	0/0
4728	2-Amino-1,3,4-thiadiazole	− (40)	− (21)	− (40)	0/0
4729	2-Acetamido-1,3,4-thiadiazole	− (125)	− (115)	+ (125)	0/0
4730	2-Ethylamino-1,3,4-thiadiazole	− (125)		+ (125)	1/1
4731	2-Allylamino-5-methyl-1,3,4-thiadiazole hydrochloride	− (500)			0/0
4900	Santonin	− (250)	− (125)		0/1
5035	Pyrogallol	− (125)	− (87.5)	− (125)	1/2
5036	Caffeine	− (300)	− (210)	− (48)	0/3
5039	Theobromine	− (500)		− (400)	0/2
5065	Nicotine	− (0.5)	− (0.35)	− (0.4)	0/0
5069	Cacothelin	− (20)		− (16)	0/0
5200	2,6-Diamino-5-phenylazo-4-pyrimidinol	± (500)		− (45)	0/0
5201	*p*-Biguanidobenzamide	− (125)			0/0
5202	Compound AA-223, Lederle				0/0
5203	4-Amino-6-*p*-chloroanilino-1,2-dihydro-2,2-dimethyl-*s*-triazine monohydrate	− (25)	− (25)	− (25)	0/0
5204	4-Amino-6-anilino-1,2-dihydro-2,2-dimethyl-*s*-triazine	− (25)	− (25)	− (25)	0/0
5205	4,6-Diamino-1,2-dihydro-2,2-dimethyl-1-(2,6-xylyl)-*s*-triazine hydrochloride				0/0
5206	4,6-Diamino-1-(*m*-bromophenyl)-1,2-dihydro-2-undecyl-*s*-triazine hydrochloride				0/0
5355	Berberine sulfate		− (25)	− (25)	0/1
5361	Ethylhydrocupreine hydrochloride (Optochin·HCl)	− (125)		− (100)	0/1
5363	Cotarnine hydrochloride	− (100)		− (60)	0/0
5364	Cinchonidine	− (500)		− (400)	0/1
5365	Strychnine	− (0.04)	− (0.02)	− (0.04)	0/1
5366	Narcotine	− (125)	− (200)	− (100)	0/2

APPENDIX—*Continued*

NSC No.	Compound name	CCBSC screening results			Other screening data
		S 180	Ca 755	L 1210	
7365	6-Diazo-5-oxo-norleucine				
9706	2,4,6-Tris(1-aziridinyl)-*s*-triazine				
9902	Ricin				0/2
10004	Quinidine sulfate	− (250)	− (200)	− (125)	0/0
11427	Codeine sulfate pentahydrate	− (150)	− (85)	− (150)	0/1
11428	Thebaine hydrochloride	− (25)	− (14)	− (12.5)	1/3
11429	Tetrahydrothebaine hydrochloride trihydrate	− (10)	− (8)	− (10)	0/0
11440	Protopine hydrochloride	− (50)	− (45)	− (50)	1/1
11441	Morphine sulfate, pentahydrate	− (75)	− (70)	− (75)	0/1
11442	Apomorphine hydrochloride	− (5)	− (4.5)	− (5)	0/1
12865	Quinine hydrobromide	− (500)	− (115)	− (125)	1/3

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